BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF SELECTED *CORTICOSTEROIDS* AND *SEX STEROIDS* AND ITS APPLICATION TO CHRONOPHARMACOKINETICS

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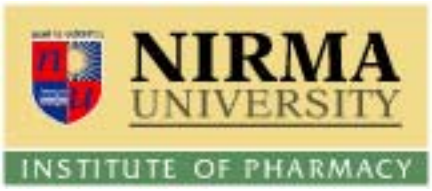

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Acknowledgments

"Each individual creature on this beautiful planet is created by God to fulfill a particular role. Whatever one achieves in life is through His help and an expression of His will. God showered His grace on me by directing towards the right path and right people. When I pay my tributes to these fine people, I am merely praising His glory."

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LIST OF ABBREVIATIONS

ACN	:Acetonitrile
ANDAs	:Abbreviated new drug applications
APCI	:Atmospheric pressure chemical ionization (APCI)
APPI	:Atmospheric pressure photo ionization
AP-1	:Activator protein-1
AUC	:Area under curve
BA	:Bioavailability
BE	:Bioequivalence
BLQ	:Below limit of quantification
BT	:Bench top
BUD	:Budesonide
CE	:Collision energy
CDER	:Center for Drug Evaluation and Research
CI	:Chemical ionization
CSF	:Cerebrospinal fluid
CSC	:Central serous chorioretinopathy
CSR	:Central serous retnopathy
CV	:Coefficient of variation
C_{max}	:Maximum serum or plasma concentration
DCM	:Dichloromethane
DE	:Dry-extract
DEE	:Diethyl ether
DEX	:Dexamethasone
EC	:Ethical committee
EDTA	:Ethylene di amine tetra acetic acid
EE	:Ethinyl estradiol
EIA	:Electroimmunoassay
ELISA	:Enzyme-linked immunosorbent assay
ESI	:Electrospray ionization
eV	:Electron volt
EXT	:Extracted
FLU	:Fluticasone propionate
FT	:Freeze thaw
GC	:Gas chromatography
GI	:Gastrointestinal
GM-CSF	:Granulocyte-macrophage colony stimulating factor

GR	:Glucocorticoid receptor
GRII	:Glucocorticoid II
HLB	:Hydrophillic-Lipophillic Balance
HPA	:Hypothalamic-pituitary-adrenal
HPLC	:High-performance liquid chromatography
HQC	:High quality control
hr	:Hour
ICF	:Informed Consent form
INDs	:Investigational new drug applications
ISTD	:Internal standard
Kel	:Elimination rate constant
LC–MS/MS	:Liquid chromatography tandem mass spectrometry
LC-UV	:Liquid chromatography-ultraviolet
LLE	:Liquid-liquid extraction
LLOQ	:Lower limit of quantification
LLOQC	:Lower Limit of Quality control
LQC	:Low quality control
LEV/LN	:Levonorgestrel
MECC	:Micellar electrokinetic capillary chromatography(
mg	:Milli gram
MIF	:Mifepristone
min	:Minute
mL	:Milli litre
MIP-1 α	:Macrophage inflammatory protein- 1 α
mm	:Milli meter
mM	:Milli molar
MQC	:Medium quality control
MRM	:Multiple reaction monitoring
MS	:Mass spectrometry
NDAs	:New drug applications
NE	:19-norethindrone
NF-kB	:Nuclear factor kappaB
ng	:Nano gram
OC	:Oral contraceptives
P-1	:Period one
P-2	:Period two
P&A	:Precision and Accuracy

pg	:Pico gram
Pgp	:P-glycoprotein
PK	:Pharmacokinetic
PPT	:Protein precipitation
PRD	:Prednisone
QC	:Quality control
Q1	:Quadrupole one
RIA	:Radioimmunoassay
RPC	:Reverse phase chromatography
TNF- α	:Tumour necrosis factor α
TRI	:Triamcinolone acetonide
SCN	:Suprachiasmatic nuclei
SD	:Standard deviation
Sec	:Second
SIL	:Stable isotopically labeled
SIM	:Selected ion monitoring
SPE	:Solid phase extraction
SRM	:Selective-reaction monitoring
STD	:Standard
S/N	:Signal-to-noise ratio
TBME	:tertiary butyl methyl ether
TFA	:Trifluoroacetic acid
TQ	:Triple quadrupole
t_{max}	:Time after administration needed to obtain maximal plasma or serum levels
ULOQ	:Upper limit of quantification
USFDA	:United States Food and Drug Administration
V_d	:Volume of distribution
WE	:Wet-extract
µg	:Micro gram
m	:Micron
°C	:Degree Celsius
%	:Percentage
r^2	:Coefficient of determination

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Chapter-1

INTRODUCTION

1.1 Bioanalysis

Bioanalytical field concerns with the analysis of drugs in the biological matrix. Literature has shown that analytical methods and techniques are continuously undergoing changes and improvements; and in many cases, they are at the cutting edge of the technology. It is also important to identify that each analytical technique has its own characteristics, which may vary from analyte to analyte. The main objective of the study also influences the technique to be used for the determination of the specific analyte [1].

A selective and sensitive bioanalytical method for the quantitative determination of drugs and their metabolites (analytes) plays a significant role in the successful conduct of bioequivalence, pharmacokinetic (PK), and toxicokinetic studies. It is essential to employ well characterized and fully validated bioanalytical methods to yield reliable results that can be satisfactorily interpreted. Published methods of analysis are often modified to suit the requirements of the laboratory developing the method for a particular analyte. The quality of these studies, which are often used to support regulatory filings, is directly related to the bioanalytical data. Therefore, the ability of bioanalytical methods to determine the concentration of parent drugs and their main metabolites is of prime importance [2].

In order to design a pertinent bioanalytical method, the analysts should initially understand the rationale of the experiment into which the analytical information will be used, requirement from the results, and effect of the results on the overall conclusion of the experiment. Once these factors are defined, the appropriate method can be developed. Once developed, and before utilization for study sample analysis, sufficient data should be obtained related to the performance of the method in order to assure its suitability for its intended use. This information is obtained by performing a method validation experiment. Bioanalytical method validation includes all procedures that demonstrate that a particular method can be used for quantitative measurement of analytes in given biological fluids, such as blood, plasma, serum, or urine, are reliable and reproducible for the intended use. Method validation experiments involve analyzing quality control samples (spiked samples) using calibration standards prepared in the same biological matrix. Setting the validation parameters acceptance is very important and should be well established [3].

The evolution of divergent analytical technologies for conventional small molecules and macromolecules, and the growth in marketing interest of macromolecular therapies, led to a special interest in bioanalytical method validation for macromolecules. Bioanalysis, perhaps more than many other types of analysis, involves challenging unknown factors that affects the analyte/s to be estimated at very low concentrations and complexity of matrices in terms of qualitative and quantitative composition. The reliability of modern methods is therefore remarkable given that analytes are often measured at concentrations even up to picogram level and even after extraction and chromatography the analyte of interest may often represent the number of ions entering the interface of an LC–MS/MS instrument[4].

Bioanalytical method development when done for the first time (for existing drugs) or a new drug entity or if new metabolites need to be identified and quantified, the plan can be divided in to,

(a) Bioanalytical method development

(b) Pre-method validation

(c) Method validation

Bioanalysis has matured significantly from early studies in drug metabolism using simple colorimetric techniques to the sophisticated hyphenated techniques which links advanced separations with mass spectrometry (MS) as detection systems, automation and robotics. The advancement of techniques resulted in the rapid emergence and widespread commercial use of "hyphenated" mass spectrometry based assays, which have largely replaced conventional high-performance liquid chromatography (HPLC), gas chromatography (GC), and GC-MS assays. Given the certainty of continued technological advances, the future will very likely bring new, even more powerful bioanalytical approaches in search for more rapid throughput and increased sensitivity [5].

Increased number of biological agents used as therapeutics has prompted the pharmaceutical industry to review and refine the aspects of bioanalytical method development and validation of these therapeutic agents in biological matrices in support of preclinical and clinical studies. These methods are used in pharmaceutical industries, pharmaceutical contract research

organizations, pharmacy laboratories and university laboratories. Same principles apply to other areas such as forensic science, toxicology and sports doping testing[6].

In recent years, liquid chromatography tandem mass spectrometry (LC-MS/MS) has emerged as the most accurate and powerful method for measuring small molecules in biological matrix due to its high sensitivity and specificity. It is a technique that combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry. Majority of the LC–MS/MS methods for quantitative steroid analysis use triple quadrupole mass spectrometry. Mass analysis is mostly performed in selective-reaction monitoring (SRM) mode which is highly selective and sensitive. Both mass analyzers are fixed on transmission of the compound-specific precursor and product ions reducing chemical noise and enhancing signal-to-noise ratio. Ionization techniques commonly used for the analysis of steroids with LC–MS/MS include electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and photo ionization (APPI). The type of ionization preferred in the different assays depends on the chemical properties of the analyte (presence of ionized or ionizable groups, polarity) and instrument characteristics. Generally, for highly polar molecules ESI is preferred, whereas for molecules with low to medium polarity APCI and APPI are used. Most steroids are measured in the positive mode. In some applications chemical derivatization is used to gain sensitivity. Derivatization can change the efficiency of the ionization, fragmentation, chromatographic retention and matrix effects [7].

LC–MS/MS has number of advantages. Besides high specificity, sensitivity and throughput, sample extraction and pre-treatment are minimized. LC-MS is frequently used in drug development at different stages including peptide mapping, glycoprotein mapping, natural products dereplication, bioaffinity screening, in vivo drug screening, metabolic stability screening, metabolite/s identification, impurities identification, degradant identification, quantitative bioanalysis, and quality control. In contrast to GC–MS, no complex, time consuming workup and derivatization of the samples is necessary [8]. Sample clean up can be reduced and analysis time can be shortened. In contrast to immunoassays, one main advantage is the possibility to measure many steroid hormones very specifically in parallel within one analysis. Furthermore, the dynamic range of the calibration spans four orders of magnitude

instead of two as for electroimmunoassay (EIA) or radioimmunoassay (RIA). Because of these characteristics, LC–MS/MS is a well suited technique for the analysis of steroid hormones. With the introduction of LC-MS, sample preparation was thought to be reduced to a minimum but this vision turned out to be obsolete and it is still necessary to draw attention for the sample preparation or to remove unwanted compounds that could interfere with the detector response.

1.2 Corticosteroids

Corticosteroids are synthetic derivatives of the natural steroid, cortisol, which is produced by the adrenal glands. Some corticosteroids however are synthesized from cholesterol within the adrenal cortex. These include glucocorticoids, which are anti-inflammatory agents with a large number of other functions, mineralocorticoids, which control salt and water balance primarily through action on the kidneys, and corticotropins, which control secretion of hormones by the pituitary gland. Corticosteroids act on the immune system by blocking the production of agents that trigger allergic and inflammatory actions, such as prostaglandins. However, they also impede the function of white blood cells which destroy foreign bodies and help keep the immune system functioning properly. The interference with white blood cell function yields a side effect of increased susceptibility to infection. Synthetic corticosteroids are used to treat various conditions, like allergic reactions, skin diseases (psoriasis, hives), breathing problems, cancer, blood disorders, eye problems, arthritis, digestive problems, and for hormone replacement. The side effects of corticosteroids include dizziness, nausea, indigestion, increased appetite, weakness or sleep disturbances, vomiting of blood, black or tarry stools, puffing of the face, swelling of the ankles or feet, unusual weight gain, prolonged sore throat or fever, muscle weakness, breathing difficulties, mood changes, vision changes and allergy[9].

Corticosteroids are drugs closely related to cortisol, a hormone which is naturally produced in the adrenal cortex (the outer layer of the adrenal gland). Commonly used corticosteroids include Budesonide, Cortisone, Dexamethasone, Hydrocortisone, Methylprednisolone, Prednisolone, Prednisone and Triamcinolone. The corticosteroids may be given orally,

injected into the vein or muscle, applied locally to the skin or injected directly into inflamed joints. These drugs are also given as eye products, inhalers, nasal drops, sprays and topical creams and ointments.

Glucocorticoids (GC) are a class of steroid hormones that bind to the glucocorticoid receptor (GR), which is present in almost every vertebrate animal cell. The name glucocorticoid derives from their role in the regulation of the metabolism of glucose, their synthesis in the adrenal cortex, and their steroidal structure. Glucocorticoids are used for the treatment of a wide range of diseases. Systemic use of glucocorticoids is often associated with significant side effects, ranging from skin fragility to full-blown iatrogenic Cushing syndrome. Prolonged use of corticosteroids leads to suppression of the hypothalamic-pituitary-adrenal (HPA)-1 axis, as a result topical corticosteroid preparations were prescribed in place of systemic corticosteroids whenever possible in the past. However, it is clear that this does not avoid all side effects. Administration of inhaled corticosteroids is also associated with dose-dependent HPA-axis suppression [10].

Establishing a definitive diagnosis of systemic corticosteroid effects and accurate monitoring requires measurement of actual concentration of the drug in serum or urine. Unfortunately, such assays are generally not available in hospital, reference, and research laboratories, and if available they may allow measurement of only a single synthetic corticosteroid. For endogenous steroids, methods based on liquid chromatography/electrospray tandem mass spectrometry have been developed. For synthetic steroids, assays have been developed specifically to analyze a single or only a few synthetic glucocorticoids and their metabolites. Developing a method for the simultaneous estimation of several synthetic corticosteroids is more challenging, owing to matrix effect and different ionization efficiencies of the analytes.

Low-dose corticosteroids may provide a favorable benefit/risk ratio for the therapeutic applications and demand quantification of the drug at very low concentrations in plasma. Developing methods with sufficient sensitivity/ selectivity for corticosteroid pharmacokinetics (PK) is a more challenging task. Furthermore, targeted therapeutic strategies involving administration by inhalation or intraocular injection could result in

extremely low but sustained systemic corticosteroid concentrations, which must be quantified to determine potential side effects [11].

1.3 Sex Steroids

Synthetic estrogens and progestins are used for hormonal contraception. Levonorgestrel/Ethinyl Estradiol is a progesterone and estrogen combination for oral contraceptives. The combined oral contraceptive has proved to be a popular, highly effective method of hormonal contraception. The combination of an orally active synthetic estrogen and gestogen suppresses gonadotrophins. Primary mechanism of this action is inhibition of ovulation; other alterations include changes in the cervical mucus (which increase the difficulty of sperm entry into the uterus) and the endometrium (which reduce the likelihood of implantation). The pattern of menstrual bleeding in the majority of cycles is highly predictable and is one of the reasons for its continuing popularity worldwide. However, the combined oral contraceptive is associated, in some women, with a number of side-effects such as acne, weight gain and menstrual irregularity, which have led to the search for newer gestogens with more favorable metabolic profiles and fewer side-effects. Rare, more serious, side-effects such as deep venous thrombosis and pulmonary embolism are thought to be associated with the estrogen component of some gestogens and have led to the development of progestogen only pills, implants and injections. The major drawback to all gestogen only methods is a high incidence of unpredictable breakthrough bleeding which leads to relatively low continuation rates [12].

Mifepristone, anti progesterone is a synthetic steroid that prevents progesterone (and glucocorticoids) from binding to hormone receptors. Mifepristone can block ovulation or retard endometrial development, depending on whether it is administered before or after ovulation. It does not activate a true biologic response to progesterone; it does, however, have both weak antiglucocorticoid and antiandrogenic activity. Mifepristone also softens and dilates the cervix, causes decidual necrosis (which leads to placental detachment), increases uterine lining prostaglandin release, increases uterine contractions, and enhances uterine sensitivity to administered prostaglandin [13].

No specific investigation of the absolute bioavailability of ethinylestradiol and levonorgestrel in humans has been conducted. However, literature shows that levonorgestrel is rapidly and completely absorbed after oral administration (bioavailability about 100%) and is not subjected to first-pass metabolism [14]. Ethinylestradiol is rapidly and almost completely absorbed from the gastrointestinal tract but, due to first-pass metabolism in gut mucosa and liver, the bioavailability of Ethinylestradiol is between 38% and 48%[15]. The concentration of Ethinylestradiol and Levonorgestrel available in the biological matrix is in pg/mL level; so a highly selective and sensitive bioanalytical method is required for their quantification.

1.4 Bioanalytical LC-MS/MS Assays

LC–MS/MS affords the specificity, precision, and limits of quantification necessary for the reliable measurement of steroids; the method requires less analysis time as compared to HPLC assays.

1.4.1 Sample pretreatment

Biological samples cannot be assayed directly, but require a pretreatment to dispose the sample of endogenous compounds, such as proteins, carbohydrates, salts, and lipids which are present in large amounts and may interfere with the analysis. In the bioanalysis of pharmaceutical compounds solid phase extraction (SPE) is a commonly used technique for sample pretreatment. SPE is a chromatographic procedure, based on the same principles as LC. Due to the wide range of cartridges and solvents that can be employed, SPE is a versatile technique. Liquid-liquid extraction (LLE) is especially suited for lipophilic compounds since the analyte transfers from the usually aqueous matrix to a nonpolar organic phase. LLE is based on the distribution of solutes between an aqueous phase, the sample to be extracted, and a water immiscible organic solvent. This procedure is followed by the evaporation of the organic phase, since these solvents cannot be directly injected onto the LC-MS system. Solvents such as hexane or ethers, often used in LLE procedures, are rapidly evaporated and, by limiting the volume of the solvent used to redissolve the residue, the sample may be concentrated [16].

Protein precipitation is the simplest means of sample pretreatment as it involves only the addition of a precipitating solvent (for instance methanol, acetonitrile, or perchloric acid solution) and subsequent homogenization and centrifugation. The clear supernatant may be injected on to the LC-MS system. Protein precipitation is one of the crudest and nonselective preparation methods; analyte concentrations should be high enough to achieve a signal that dominates the signal of the endogenous material for accurate determination. Protein precipitation, however, is not always sufficient since it leaves many matrix constituents in the sample that may interfere with the assay [17]. Due to the selectivity of MS detectors it was believed that sample pretreatment for LC-MS/MS assays was redundant. Although sample pretreatment for LC-MS/MS assays does not need to be as elaborate as for other LC based assays especially those utilizing UV detection, it remains pivotal to remove matrix components that may contaminate the system or cause ion suppression when high sensitivity is desired. Some matrices do not require an elaborate sample pretreatment since they consist mainly of water, such as cerebrospinal fluid (CSF), tear fluid, and even urine. Nevertheless, the extent of sample pretreatment needed will become evident while setting up the assay. With decreasing LC run times sample pretreatment has become the rate-limiting step in bioanalytical assays. In order to reduce the time to prepare a sample several forms of automation have emerged in the field of bioanalysis. Automated sample pretreatment or on-line sample pretreatment may be a suitable means of time reduction for sample preparation as long as the procedure ensures an adequate removal of interfering matrix components.

1.4.2 Chromatography

LC is one of the most widely used bioanalytical chromatographic techniques. A variety of mobile and stationary phases are commercially available to analyze a large range of compounds. This versatility is often not found in GC, since the mobile phase cannot be varied. Contrary to most LC-UV assays, in bioanalytical assays LC-MS/MS chromatography is mainly used to separate the analytes from matrix components and not from other analytes, internal standards, or potential metabolites. Co-eluting peaks do not interfere with a correct analysis of the analyte due to the selectivity of the MS. High levels of matrix components, however, may cause ion suppression and therefore need to be separated from the analyte[18] .

Short LC columns are usually sufficient to obtain this separation and thus run times can be reduced substantially. MS detection is not compatible with each solvent or eluent additive that are commonly used in assays employing LC separation. Normal phase or ion-exchange chromatography for instance can usually not be employed in combination with MS, since their eluents (organic solvents, salts and other additives) are not compatible with the MS. Furthermore, mobile phase additives such as phosphates are undesirable since they contaminate the MS and volatile strong acids such as trifluoroacetic acid (TFA) commonly used as ion-pairing agent for chromatography of peptides and proteins, reduce the MS signal dramatically. In LC-MS, reversed phase chromatography is almost exclusively used in combination with eluents that consist of water, methanol, or acetonitrile. Ammonium acetate, ammonium formate, acetic acid, and formic acid are the most commonly used additives for positive ionization LC-MS/MS [19]. Ammonium hydroxide may be used for assays employing negative ionization. The LC system is miniaturized by using analytical columns with smaller internal diameters (i.d., 2.1, 2.0 and 1.0 mm) and lower flow rates (<200 μL/min). Since the MS is a concentration sensitive detector, the resulting decrease in the amount of sample introduced into the MS does not necessarily influence the sensitivity of the assay.

1.4.3 Detection

A mass spectrometer is a selective and sensitive LC detector, suitable for the detection of a large range of compounds, provided that the compound can be ionized. The response of MS depends on a compound's molecular structure and may vary due to several instrument related parameters and experimental conditions. These variations can be corrected by using an internal standard. The most appropriate internal standards for MS are stable isotopically labeled (SIL) internal standards or structural analogues of the analyte, since these compounds are expected to possess similar ionization efficiencies as the analyte. For quantitative assays employing MS detection triple quadrupole (TQ) systems are most commonly used. When a TQ is operated in the multiple/selective reaction monitoring (MRM/SRM) mode the analyte is identified and detected not only by means of its molecular ion but also by means of a typical fragment ion, obtaining higher sensitivity and superior selectivity compared to any other MS system [20].

1.5 Bioanalytical Method Validation

Following the development of a bioanalytical LC-MS/MS assay and before implementation into clinical pharmacological studies, preclinical evaluation and routine use, it needs to be validated. Validation is essential to ensure the accuracy and precision of the acquired data. In 2001, the United States Food and Drug Administration (USFDA) published guidelines for the validation of bioanalytical assays, which are considered to be the standard for validation parameter assessments and requirements [1]. The guidelines not only apply to bioanalytical procedures such as GC and LC based assays (including GC-MS, LC-MS, GC-MS/MS, and LC-MS/MS), but also immunological and microbiological procedures for the quantitation of drugs and/or metabolites. The fundamental parameters for validation as laid by USFDA include: (1) Calibration/Standard curve (2) accuracy & precision, (3) selectivity/specificity (4) recovery and (5) stability. Validation involves documenting, through the use of specific validation experiments, that the performance characteristics of the method are suitable and reliable for the intended analytical applications. The acceptability of analytical data corresponds directly to the criteria used to validate the method.

1.5.1 Calibration/Standard Curve

A calibration (standard) curve is the relationship between instrument response and known concentrations of the analyte. A calibration curve should be generated for each analyte in sufficient number of representative samples. A sufficient number of standards should be used to adequately define the relationship between concentration and response. A calibration curve should be prepared in the same biological matrix as the samples in the intended study by spiking the matrix with known concentrations of the analyte. The number of standards used in constructing a calibration curve will be a function of the anticipated range of analytical values and the nature of the analyte/response relationship. Concentrations of standards should be chosen on the basis of the concentration range expected in a particular study. A calibration curve should consist of a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard), and six to eight non-zero samples covering the expected range, including lower limit of quantification (LLOQ) [21].

1.5.2 Accuracy and Precision

The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. Accuracy should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The mean value should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%. The deviation of the mean from the true value serves as the measure of accuracy.

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. Precision should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV. Precision is further subdivided into within-run, intra-batch precision or repeatability, which assesses precision during a single analytical run, and between-run, inter-batch precision or repeatability, which measures precision with time, and may involve different analysts, equipment, reagents, and laboratories [1, 4].

1.5.3 Selectivity/Specificity

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. For selectivity, analyses of blank samples of the appropriate biological matrix (plasma, urine, or other matrix) should be obtained from at least six sources. Each blank sample should be tested for interference, and selectivity should be ensured at the lower limit of quantification (LLOQ). Potential interfering substances in a biological matrix include endogenous matrix components, metabolites, decomposition products, and in the actual study, concomitant medication and other exogenous xenobiotics. If the method is intended to quantify more than one analyte, each analyte should be tested to ensure that there is no interference [1, 22].

1.5.4 Recovery

The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. Recovery pertains to the extraction efficiency of an analytical method within the limits of variability. Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise, and reproducible. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with unextracted standards that represent 100% recovery [1].

1.5.5 Stability

Drug stability in a biological fluid is a function of the storage conditions, the chemical properties of the drug, the matrix, and the container system. The stability of an analyte in a particular matrix and container system is relevant only to that matrix and container system and should not be extrapolated to other matrices and container systems. Stability procedures should evaluate the stability of the analytes during sample collection and handling, after long-term (frozen at the intended storage temperature) and short-term (bench top, room temperature) storage, and after going through freeze and thaw cycles and the analytical process. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis. The procedure should also include an evaluation of analyte stability in stock solution. All stability determinations should use a set of samples prepared from a freshly made stock solution of the analyte in the appropriate analyte-free, interference-free biological matrix. Stock solutions of the analyte for stability evaluation should be prepared in an appropriate solvent at known concentrations [4,22].

1.6 Chronopharmacokinetics

Many variables are involved in kinetic studies related to the patients, the drug and/or its environment. Some of these factors include age, gender, diseases, pathophysiological conditions, etc. These factors have been extensively studied and are regularly mentioned and reviewed in numerous articles. Differences between morning and evening meal conditions in

our daily life play a major role in mechanism underlying the circadian changes of drug absorption in man [23]. The time dependent changes in kinetics may proceed from the circadian variation at each step, of drug absorption distribution, metabolism and excretion. Nevertheless, if time of administration of drugs is taken into account to determine the relationships between time and concentrations in usual pharmacokinetic studies, the possible influence of time of drug administration is rarely taken into account [24].

Chronopharmacokinetics deals with the study of the temporal changes in absorption, distribution, metabolism and elimination of the drugs varying throughout the day. This field in biomedical and pharmaceutical research investigates: (1) variations in the effects of drug as a function of time of drug administration; (2) mechanisms involved in temporal variations of drug effectiveness and drug toxicity; (3) the application of the biological rhythms in therapeutics. Thus, the pharmacokinetic parameters characterizing these different steps, conventionally considered to be constant in time, depend on the moment of drug administration. Time of day has to be regarded as an additional variable influencing the kinetics of a drug [25].

Decrease or increase in the rate of these pharmacokinetic variables are not randomly distributed along the 24 hour scale; on the other hand their timings corresponds to a highly ordered temporal organization controlled by a set of pacemakers which are probably interconnected and integrated hierarchically. As a consequence, the kinetics of a drug do differ from one time of drug administration to another and thus circadian variations in the kinetics or effects of drugs cannot be explained solely by variations in external factors such as presence of food in the stomach, diseases and drug interactions.

Temporal changes can be involved at each step in the sequence of pharmacokinetic processes: temporal variations in drug absorption from the gastro-intestinal tract (due to circadian variations in gastric acid secretion and pH, motility, gastric emptying time, gastrointestinal blood flow), plasma protein binding and drug distribution, drug metabolism (temporal variations in enzyme activity, hepatic blood flow) and in renal drug excretion (due to variation in glomerular filtration, renal blood flow, urinary pH and tubular resorption). Thus, the time of administration of a drug is an important source of variation which must be taken into

account in kinetic studies and particular aspects of chronopharmacokinetics are needed. The main aim of chronopharmacokinetic studies is to control the time of administration which among others, can be responsible for variations of drug kinetics but also may explain chronopharmacological effects observed with certain drugs. Studies in animals and humans have been indicated that C_{max} (maximum serum or plasma concentration), t_{max} (time after administration needed to obtain maximal plasma or serum levels), AUC (area under curve) and half life ($t_{1/2}$) could vary as function of time of administration of the drug [26, 27].

Chronopharmacokinetic study is much needed when daily variations in pharmacokinetics may be responsible for time dependent variations in drug effects (e.g. some antimicrobial agents are more effective at a specific time of day), drugs have a narrow therapeutic range, symptoms of a disease are clearly circadian phase-dependent (e.g. nocturnal asthma, angina pectoris, myocardial infarction, ulcer disease) and drug plasma concentrations are well correlated to the therapeutic effect with circadian rhythm. Despite growing interest in pharmacokinetics, the time of administration of a drug is often neglected in kinetic studies. Data from chronopharmacological studies now clearly demonstrate that lack of consideration of such factors may lead to a possible supplementary source of variability. The knowledge of the risk of a disease during the circadian rhythm, evidence of 24 hrs rhythm dependencies of drug pharmacokinetic, effects, and safety constitutes the rationale for pharmacotherapy. In order to monitor the rhythmic changes it will be very useful to choose the most appropriate time of the day for administration of the drugs that may increase their therapeutic effects and/or reduce their side effects. It is necessary to understand how biological rhythms interfere with drug kinetics with respect to time of administration of a drug.

Chapter-2

LITERATURE REVIEW

A selective and sensitive bioanalytical method for the quantitative determination of drugs and their metabolites (analytes) plays a significant role in the successful conduct of pharmacokinetic (PK), toxicokinetic and bioequivalence studies. A scientifically validated method is essential to rule out unreliable results which may lead to erroneous estimation of effects, to false interpretations, and to unjustifiable conclusions [1, 4].

Bioanalytical method validation includes all procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological fluid, such as blood, plasma, serum, or urine, are reliable and reproducible for the intended use. Therefore, new analytical methods to be used in clinical and/or forensic toxicology require more attention towards method development as the quality of a bioanalytical method largely depends on method development. The guidelines related to the bioanalytical method validation describe basic terminologies, but do not suggest a validation strategy to evaluate the performance of a method [3]. However, validation can objectively demonstrate the inherent quality of a method by fulfillment of acceptance criteria as per the method validation guidelines and thus prove its applicability for its intended purpose [1, 4, 6].

Literature has shown increase in the parameters of method validation and tightened acceptance criteria. This research work, explains all the procedures used to validate bioanalytical methods, which can be used for the research phase of drug discovery, pharmacokinetic, toxicokinetic and metabolic studies in animals and clinical studies.

2.1 Method validation and validation parameters

The word validation originates from the Latin "*validus*" meaning strong, and suggests something has been proved to be true, useful and of an acceptable standard. Based on the importance of method validation in the bioanalytical field, a number of guidance documents on this subject have been issued by various international organizations and conferences [5]. All guidelines provide assistance to sponsors of investigational new drug applications (INDs), new drug applications (NDAs), abbreviated new drug applications (ANDAs), and supplement in developing bioanalytical method validation information used in human clinical pharmacology, bioavailability (BA), and bioequivalence (BE) studies requiring pharmacokinetic (PK) evaluation [20]. These guidelines also apply to bioanalytical methods used for non-human pharmacology/toxicology studies and preclinical studies.[1] Several key documents, journal publications and scientific meeting presentations are available to

bioanalytical laboratories to guide and assist the development and validation of bioanalytical methods.

Most widely employed bioanalytical techniques include, but are not limited to, conventional chromatography based methods (such as GC and HPLC), mass spectrometry based methods (such as GC-MS and LC-MS), and ligand-based assays (such as radioimmunoassay [RIA] and enzyme-linked immunosorbent assay (ELISA). Many of the principles, procedures, and requirements for quantitative bioanalytical method validation are common to all types of analytical methodologies. Liquid chromatography-mass spectrometry (LC-MS/MS) is an analytical technique that couples high resolution chromatographic separation with sensitive and specific mass spectrometric detection. The key elements of quantitative bioanalytical parameters essential to ensure the acceptability of the performance of a bioanalytical method are system suitability, linearity, accuracy, precision, selectivity, sensitivity, reproducibility, ruggedness and stability.

2.1.1 System suitability

System suitability is widely recognized as a critical component of bioanalysis which is performed prior to any analytical run to ensure that the LC-MS/MS system is performing in a manner that leads to the generation of accurate and reproducible data [28].

2.1.2 Specificity/Selectivity

Selectivity/specificity will be assessed to show that the intended analytes are measured and that their quantitation is not affected by other components of biological matrix, metabolites, degradation products, or co-administered drugs. Specificity should be determined for each analytes in the assay. After an extensive literature review of papers on chromatographic method validation published in analytical journals revealed that selectivity is measured by comparing chromatograms of extracted blank samples without analyte, with extracted analyte samples at LLOQ level. The interference observed at the retention time of analyte in blank sample should be $\leq$ 20% of extracted LLOQ sample [1, 22].

2.1.3 Sensitivity or Lower Limit of Quantification

The lower limit of quantification (LLOQ) is defined as the lowest concentration or amount of analyte that can be determined with an acceptable level of precision and accuracy. LLOQ is estimated based on the accuracy, precision and signal-to-noise ratio (S/N) [1].

2.1.4 Carryover

Eliminating carryover from a bioanalytical method is a time and resource consuming process. It is necessary to investigate root causes for carryover. Absolute elimination of carryover may not be practically possible. In HPLC analyses the appearance of an analyte of interest when a blank sample is injected is an undesirable state. Peaks which appear in blank samples may be caused by analyte retained from previous injections (carryover), or non-analyte related peaks which can arise either from a previous injection (late eluters) or the current injection (interfering endogenous peaks). Sample carryover is a major problem that can influence the accuracy and precision of high performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) with the consequences being more prominent at lower concentrations. The continuous increase in sensitivity of new-generation LC-MS/MS instruments, with detection limits going as low as picogram or femtogram range and the possibility of using wider calibration ranges, has also drastically increased the risk of carryover during bioanalysis. As per the guidelines any resulting peak in the blank should have an area less than 20% of the lower limit of quantitation (LLOQ). Carryover can add significant time to the method development process as well as the analyte run time and reduce productivity [29,30,31]

2.1.5 Matrix effect

Phospholipids, in particular glycerophosphocholines and lysophosphatidylcholines, represent the major class of endogenous compounds causing significant matrix effects. Phospholipids are an important class of biological compounds containing one or more phosphate groups. The molecular structure of phospholipids exhibit two major functional group regions: a polar head group substituent, which includes an ionizable organic phosphate moiety as well as other polar groups of various types, and one or two long chain fatty acid ester groups, which impart considerable hydrophobicity to the molecule. In particular, the highly ionic nature of phospholipids makes them responsible for influencing the ionization in electrospray MS sources and desolvation of the LC effluent droplets in electrospray MS analysis. Therefore, the removal of phospholipids represents an extremely important step in the sample preparation process. Suppression or enhancement of analyte ionization by co-eluting compounds mainly

depend on the sample matrix, the sample preparation procedure, the quality of chromatographic separation, mobile phase additives, and ionisation type.

It is obvious that ion suppression, as well as ion enhancement, may affect validation parameters such as specificity and selectivity. Studies of ion suppression/ enhancement are integral part of the validation of any analyte using LC–MS/MS method. Matrix effect is assessed by the post-column infusion method and the post-extraction spike method. Matrix effects may be reduced by injection of smaller volumes or dilution of the samples (useful as long as instrumental sensitivity remains adequate), optimization of sample preparation method and/or optimization of chromatographic conditions. If sensitivity is not an issue, APCI or electron ionization source, can be used because these are less sensitive to matrix effects. In common industrial practice matrix effect is reduced by working on sample preparation technique, chromatographic conditions, mass spectrometric conditions and by using appropriate internal standard [17,18,19].

2.1.6 Calibration range and Linearity

The selection of the calibration range for bioanalytical method is based on the expected concentration in the invivo study samples. The calibration range should not be too wide, as the accuracy and precision will suffer at the extremes of the range. Neither should it be too narrow, as this will invariably mean, introduction of dilution steps for pharmacokinetic studies, when concentrations exceed the upper limit of the linearity range. Least-square linear regression is normally used to mathematically define the calibration line. It is a general policy not to include the blank standard in the calibration, and not force the calibration through zero. In general, a weighting factor (usually *1/*x or $1/x^2$) is used to avoid biasing the calibration line in favor of the higher standards [4, 21].

2.1.7 Accuracy and precision

Accuracy is defined as the closeness of agreement between the test result and the accepted reference value. It is determined by calculating the percent difference between the measured mean concentrations and the corresponding nominal concentrations. Precision is ‘‘the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. The determination of this parameter is one of the basic steps in the process of achieving

repeatability and reproducibility. Assessing the precision implies expressing numerically the random error or the degree of dispersion of a set of individual measurements by means of standard deviation and coefficient of variation. Accuracy and precision of the assay should be determined for both intra and inter analytical runs. FDA guidance for validation of bioanalytical methods suggests evaluating the accuracy by measuring a minimum of three concentration levels prepared in five replicates in the range of expected concentrations [1].

2.1.8 Stability

Stability of the analyte during the whole analytical procedure is a prerequisite for reliable quantification. Method validation must include stability experiments for the various stages of analysis including storage prior to analysis. The stability of the stock solutions of drug and internal standards should be evaluated at room temperature and under refrigerated conditions. Long-term stability of the sample in matrix should be established under storage conditions, to confirm analyte/s stability in the test system matrix covering the length of time from sample collection to sample analysis. Since samples are often frozen and thawed, freeze/thaw stability of analytes should also be evaluated. Bench-top stability is the stability of analyte under the conditions of sample handling (e.g. ambient temperature over time needed for sample preparation). Instability cannot only occur in the sample matrix, during sample processing as well as after sample processing. It is therefore important to test the stability of an analyte in various steps of extraction like stability in extraction solvent, buffer/reagent, stability of analyte after evaporation and after reconstitution/elution in the prepared samples under conditions of analysis (e.g. auto sampler conditions). Extraction of the samples from plasma involves different steps. During each step of extraction, samples will be exposed to different conditions and consume some amount of time. In order to ensure the stability of analyte/s during each step of the sample processing, process stability should be performed for the anticipated time of storage at different stages of sample processing [1,7,22].

2.1.9 Reinjection Reproducibility

Reinjection reproducibility is assessed in order to determine the possibility of reinjection of processed samples, in the event that their initial analysis is interrupted because of instances like instrument failure. The samples are subjected to an initial instrumental analysis and after a few hours same samples are reanalyzed. Results of the reanalyzed samples are compared with that of the initial analysis [4,7].

2.1.10 Recovery

The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. Recovery pertains to the extraction efficiency of an analytical method within the limits of variability. Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise, and reproducible [1].

2.1.11 Ruggedness

Ruggedness of an analytical procedure evaluates the consistency of the results with variations in external factors such as analyst, instruments, change in solvent/reagent make and change in lot number of columns. By considering these critical external factors, it is evident that ruggedness is a measure of reproducibility of test results under normal, expected operational conditions from laboratory to laboratory and from analyst to analyst [32].

2.1.12 Dilution Integrity

In preclinical and clinical evaluation, when some sample concentrations exceed the upper limit of quantification and if insufficient sample volume is available for processing, a test for sample dilution with blank matrix during validation should be performed. One or more additional QC samples at concentrations higher than the upper limit of the calibration curve should be prepared, covering the maximum expected concentration. These QC samples are diluted with blank matrix to bring the concentration within the calibration range and then analyzed [4].

2.2 Corticosteroids

Corticosteroid drugs are either synthetic or naturally occurring substances with the general chemical structure of steroids. They are used therapeutically to mimic the effects of the naturally occurring corticosteroids. Glucocorticoids produce their effect on responsive cells by activating the glucocorticoid receptor (GR) to directly or indirectly regulate the transcription of target genes.(*figure no.01*) However, the major mechanism by which GR switches off inflammatory genes is by binding as a monomer either directly, or indirectly, with the transcription factors activator protein-1 (AP-1) and nuclear factor kappaB (NF-kB), which are up-regulated during inflammation, thereby inhibiting the pro-inflammatory effects of a variety

of cytokines, including interleukin (IL)-1b, IL-4, IL-5, IL-8, granulocyte-macrophage colony stimulating factor (GM-CSF), tumour necrosis factor α (TNF- α), and macrophage inflammatory protein- 1 α (MIP-1 α). By contrast, gene induction events mediated by a GR homo dimer are responsible for many side effects of corticosteroids [9].

Synthetic corticosteroids are used to treat various conditions, ranging from brain tumors to skin diseases. Glucocorticoids are important medications prescribed for the treatment of joint pain or inflammation (arthritis), temporal arteritis, dermatitis, allergic reactions, asthma, hepatitis, systemic lupus erythematosus, inflammatory bowel disease (ulcerative colitis and Crohn's disease), sarcoidosis and for glucocorticoid replacement in Addison's disease or other forms of adrenal insufficiency . Topical formulations are also available for the skin, eyes (uveitis), lungs (asthma), nose (rhinitis), and inflammatory bowel disease. Corticosteroids are also used supportively to prevent nausea, often in combination with 5HT3 antagonists [33].

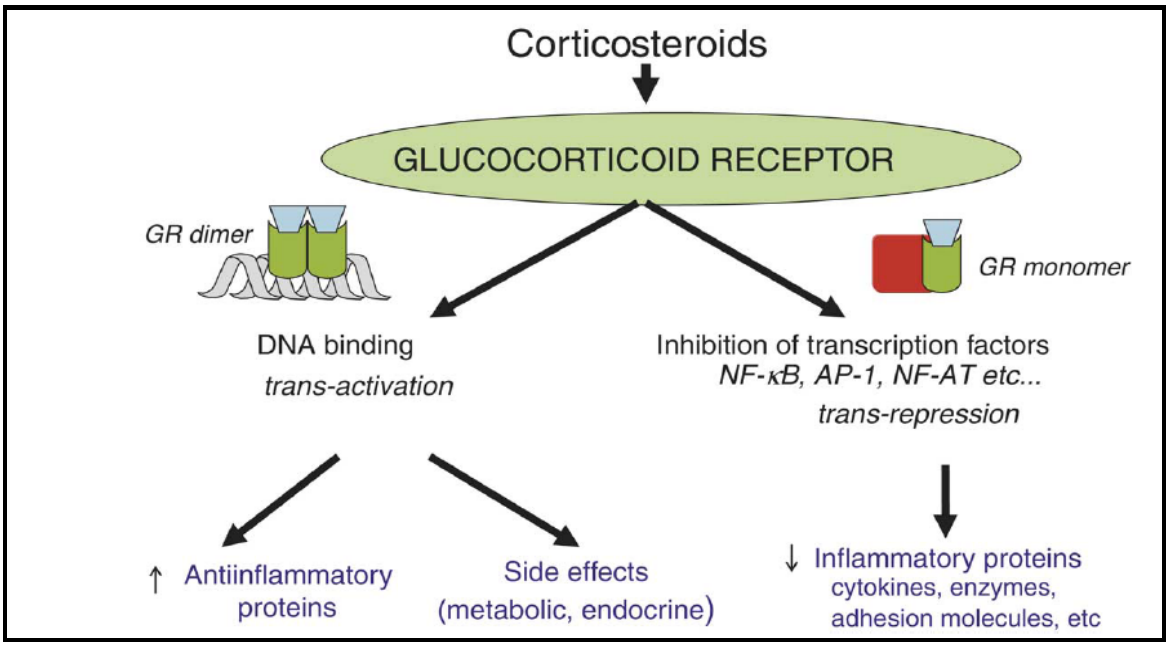

Figure No.01. Mechanism of action of corticosteroids

Corticosteroids are very powerful drugs that affect the entire body; even corticosteroids used on large areas of skin for long periods are absorbed in sufficient quantity to cause systemic side effects. Many of the synthetic corticosteroids, such as prednisone, prednisolone, triamcinolone, and dexamethasone, are more potent than the naturally occurring compounds. Budesonide and fluticasone propionate are the most potent inhaled glucocorticosteroids

available in the market. One of the main undesired effects of glucocorticoids is drug-induced Cushing's syndrome. Typical side effects attributed to mineralocorticoid activity include hypertension, hypokalemia, hypernatremia without causing peripheral edema, metabolic alkalosis and connective tissue weakness. There may also be impaired wound healing or ulcer formation because of the immunosuppressive effects. Clinical and experimental evidence indicates that corticosteroids can cause permanent eye damage by inducing central serous retinopathy (CSR, also known as central serous chorioretinopathy, CSC). A variety of steroid medications, from anti-allergy nasal sprays (Momentasone furoate) to topical skin creams, to eye drops have been implicated in the development of CSR [10].

Literature search on recent studies show low-dose corticosteroids to provide a more favorable risk/benefit ratio than higher doses for a number of clinical applications. [11,34] These low dose regimens ultimately result in very low concentration of corticosteroids in body fluids as low as pg/mL in plasma[31-33] and the PK analysis necessary to manage these therapies is hampered by the inadequate sensitivity of the most established analytical methods. In addition to this scenario, certain drug delivery strategies, such as inhalation or intraocular injection, also result in low, sustained systemic drug concentrations, often too low to be detected by current methods. Because of the pleiotropic pharmacological effects of the corticosteroids, and the high potency of some of these agents, systemic levels must be determined to ensure safety of the therapy. Finally, administration of some corticosteroid prodrugs, such as acetates/propionates, often results in sustained, low concentrations in plasma.[35] Therefore, a highly sensitive and selective analytical approach is necessary to determine the sustained low concentrations of corticosteroids that may be present systemically or in tissues. The reported methods for the determination of synthetic steroids have been developed specifically to analyze a single or only a few synthetic glucocorticoids and their metabolites.

Prednisolone (PRE): Prednisolone is designated chemically as pregna-1,4-diene-3,20-dione,11,17,21-trihydroxy-,(11β)-. The molecular formula is $C_{21}H_{28}O_5$, having a molecular weight of 360.45. Structure is presented in *figure no.02 (b).*

Prednisone (PRD): Prednisone (pregna-1,4-diene-3,20-dione,11,17,21-trihydroxy-,(11β)-) is a synthetic corticosteroid used as anti-inflammatory and immunosuppressive agent.[36] Its chemical formula is $C_{21}H_{26}O_5$ with a molecular weight of 358.43. Structure is presented in *figure no.02 (a).*

Prednisone is metabolized in the liver and intestine to its active form, prednisolone, by the enzyme 11β-hydroxysteroid dehydrogenase which is also responsible for the inactivation of prednisolone by converting it back into prednisone. One of the adverse events of prednisolone is inhibition of the hypothalamic pituitary adrenal (HPA) axis, resulting in inhibition of the production of cortisol, which serves as a biomarker for the activity of prednisolone [37].Prednisone, its metabolite prednisolone and the endogenous cortisol and cortisone are very similar in structure, hence the analytical methods should be selective enough to distinguish between the synthetic and the endogenous corticosteroids. The reported analytical methods have employed a variety of techniques such as radioimmunological or competitive protein binding assays [38], micellar electrokinetic capillary chromatography (MECC) using bile salts for the simultaneous determination of six corticosteroids, including betamethasone, cortisone, prednisolone, 6α-methylprednisolone, triamcinolone, and prednisone[39]. High performance liquid chromatography (HPLC) with UV detection methods [40] require greater separation time. Jusko W.J. et al. have reported HPLC-UV method for the simultaneous determination of prednisone, cortisol and prednisolone in human plasma with the detection limits of 10 ng/mL and 5 ng/mL respectively. But this method utilizes carcinogenic solvents for sample extraction [41]. HPLC with fluorimetric detection required precolumn derivatization for the conversion of corticosteroids into the corresponding glyoxal compounds.The extracts were derivatized by reaction with 1,2-diamino-4,5- methylenedioxybenzene, a fluorogenic reagent for dicarbonyl compounds. However, this was a very time consuming method [42]. Liquid chromatography coupled to mass spectrometry detection has been shown to produce low ng/mL quantitation of glucocorticoid mixtures in urine [43] and for prednisone, cortisol, dexamethasone and prednisolone in serum [44] and plasma [45].

Radioimmunoassay methods are usually impacted by cross reactivity, thus lacking adequate selectivity between cortisol and prednisolone. HPLC methods with UV detection have sensitivity shortfalls due to the inherent limitations of UV detectors which cannot measure concentrations less than ng/mL. Hence sample extraction methods require relatively high volumes of matrix and extensive cleanup procedures. Moreover, HPLC methods require the use of analogue internal standards, which must be chromatographically separated from the compounds of interest, resulting in increased run times. GC–MS and fluorescence methods employ derivatization steps which are time consuming. Recent developments in analytical

techniques have lead to the large-scale use of mass spectrometers with the clear advantage of increased sensitivity and added selectivity, which in most cases translates into the ability to bypass the need for chromatographic separation.

Budesonide (BUD): Budesonide is a synthetic corticosteroid, designated chemically as (RS)-11β, 16α, 17,21-tetrahydroxypregna-1,4-diene-3,20-dione cyclic 16,17-acetal with butyraldehyde. The molecular formula of budesonide is $C_{25}H_{34}O_6$ and its molecular weight is 430.5. Structure is presented in *figure no.02(c).*

Budesonide is a potent glucocorticosteroid with high local anti-inflammatory but low systemic glucocorticoid activity [46]. Its pharmacokinetic characteristics of low oral bioavailability, large volume of distribution, and high systemic clearance lead to low plasma concentrations following inhalation of therapeutic doses. The pharmacokinetic profile of BUD was previously determined in dogs and a small number of human subjects by giving radiolabelled BUD intravenously using high performance liquid chromatography (HPLC)[47]. Radioimmunoassays combined with liquid chromatography (RIA– LC) have been used for determination of BUD in human plasma [48] with LLOQ of 0.133 ng/mL. Published methods for quantification of BUD in human plasma involve an automated liquid chromatography thermospray mass spectrometry (LC–TSP/MS)[49] and liquid chromatography atmospheric pressure chemical ionization tandem mass spectrometry (LC–APCI-MS–MS).[62] The LC–TSP/MS method displayed widely variable thermospray responses from one compound to another.

Fluticasone propionate (FLU): Fluticasone propionate is a glucocorticoid chemically identified as S-(fluoromethyl) 6α,9-difluoro-11β,17 dihydroxy-16α-methyl-3-oxoandrosta-1,4-diene-17β-carbothioate, 17-propionate, molecular weight of 500.6; the molecular formula is $C_{25}H_{31}F_3O_5S$. Structure is presented in *figure no.02(d).*

The structure of Fluticasone propionate is based on androstane, rather than pregnane, corticosteroid nucleus. The molecule is designed to maximize topical anti-inflammatory activity and minimize the unwanted systemic effects associated with other glucocorticoids like suppression of hypothalamopituitary-adrenal axis. At clinical doses of inhaled glucocorticoids it is estimated that only 20% of the drug reaches the lung with the majority of drug being swallowed and entering the gastrointestinal (GI) tract. FLU is poorly absorbed from the GI epithelium and under-goes extensive first pass metabolism to 17β-carboxylic acid. The oral

bioavailability is less than 1%. While advantageous in inhalation therapy, this results in low plasma concentrations; any detectable systemic levels are due to adsorption from the lungs [50]. This leads to a significant challenge in developing sensitive methods required to generate pharmacokinetic data following therapeutic doses.

Due to lack of a suitable chromophore or fluorophore in the molecule and the suitability of steroid molecules for raising high affinity, selective antibodies, the first method developed for FLU was a radioimmunoassay (RIA). It was necessary to further enhance the method by the inclusion of a solid-phase extraction (SPE) step prior to the RIA in order to provide pharmacokinetic information after inhaled doses. This extended the LLOQ to 50 pg/mL but at the same time reduced the sample throughput [51]. LC–MS/MS after solid-phase extraction with positive APCI detection method has been reported with the sensitivity (LLOQ) upto 200 pg/mL[52]. Li et al. have reported a method for FLU in human plasma using a combination of protein precipitation and solid-phase extraction followed by LC-APCI-MS/MS with a lower limit of quantification of 200 pg/mL [53] and 20 pg/mL [54] from a sample size of 1 mL of plasma. All the reports of HPLC–MS/MS techniques use solid-phase extraction, and most of them employ Atmospheric Pressure Chemical Ionization (APCI).A liquid chromatographic, tandem mass spectrometric method (LC-MS/MS) with automated solid phase extraction (SPE) was developed and validated with a lower limit of quantitation (LLOQ) of at least 25 pg per mL [55].

Dexamethasone (DEX): Dexamethasone is 9α-fluoro-16α-methyl-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione, a synthetic derivative of the glucocorticoid hydrocortisone used as a potent anti-inflammatory agent. The molecular formula is $C_{22}H_{29}FO_5$. The molecular weight is 392.47 and structure is presented in *figure no0.2 (e).*

Numerous chromatographic methods have been reported for the determination of dexamethasone in biological matrices based on high-performance liquid chromatography (HPLC) with detection by ultraviolet spectroscopy [56], radioimmunoassay [57] and HPLC-radioimmunoassay[74].Wu et al have reported a method for the determination of dexamethasone in plasma by HPLC[58] with fluorogenic derivatization. A GC–MS method has been reported for the detection of dexamethasone in urine [59], which required derivatization prior to analysis. Antignac JP et al. have reported detection of corticosteroid residues in biological samples (urine, hair, muscle) using high performance liquid

chromatography/tandem mass spectrometry [60]. Sensitive methods have been published by Bagnati et al. with LLOQ of 146 ng/mL[61], where an "on-line" immunoaffinity- HPLC system was used as clean up prior to GC–MS detection. In recent years, high-performance liquid chromatography coupled with electrospray MS detection technique has been widely applied for the detection of dexamethasone using LC-tandem mass spectrometry in different biological fluids like sheep plasma [62], bovine liver[63] ,atmospheric pressure chemical ionization in bovine plasma, tissues[64] and rabbit ocular matrices. DiFrancesco et al. have reported liquid chromatography coupled to tandem mass spectrometry for the determination of different glucocorticoids, in which the reported LLOQ was 0.7 ng/mL [65]

Triamcinolone acetonide (TRI):Triamcinolone acetonide, (8S,9R,10S,11S, 13S,14S, 16R, 17S)-9-fluoro-11,16,17-trihydroxy-17-(2-hydroxyacetyl)-10,13-dimethyl-6,7,8,11,12,14,15, 16- octahydrocyclopenta[a]phenanthren-3-one) is a corticosteroid with a molecular weight of 434.51, chemical formula $C_{24}H_{31}FO_6$ and structure is presented in *figure no.2(f).*

Triamcinolone acetonide is a potent glucocorticoid, used for anti-inflammatory treatment in patients with bronchial asthma or rheumatoid arthritis [66]. TRI acts by inhibiting phospholipase A2 induction through lipocortin production [67]. Corticosteroids have also been shown to down regulate many inflammatory mediators including multiple interleukins, prostaglandins and tumor necrosis factor. Triamcinolone acetonide is an adduct of triamcinolone and acetone, but not a pro-drug of triamcinolone. Derivative spectrophotometry has been applied extensively to the simultaneous determination of substances with overlapping spectra, which is frequently made on the basis of zero-crossing measurements.[68] Reported HPLC methods for the determination of triamcinolone acetonide in human plasma and urine have been published[66,69,70]. Some RIA assays have been established, but lack specificity and validation details are not reported[71]. GC methods for the determination of triamcinolone acetonide in urine samples have also been published [72]. The limit of quantification for triamcinolone acetonide in human plasma by HPLC method was reported to be 0.6 ng/mL[66].These methods do not provide any data related to the stability of triamcinolone acetonide in plasma. Degenring et al. have reported a HPLC-tandem mass spectrometry method using serum samples with LLOQ of 0.73µg/mL[70]. The method was found to be time consuming and lacked sensitivity.

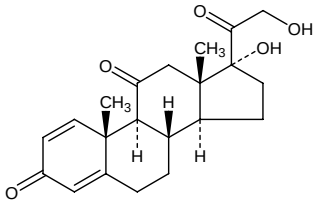

Figure No.02(a) Structure of Prednisone

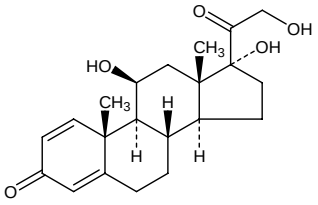

Figure No.02(b) Structure of Prednisolone

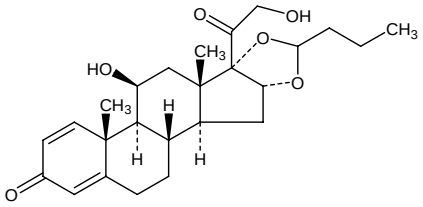

Figure No.02(c) Structure of Budesonide

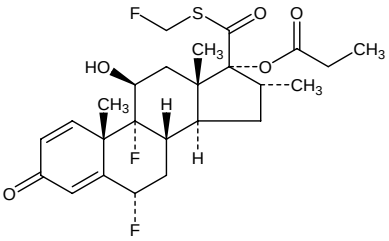

Figure No.02 (d) Structure of Fluticasone-propionate

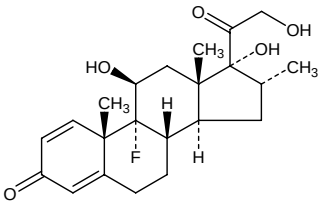

Figure No. 02(e) Structure of Dexamethasone

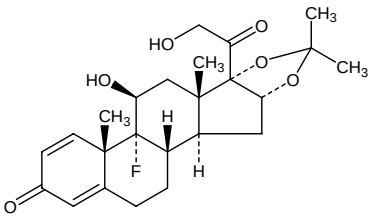

Figure No.02 (f) Structure of Triamcinolone acetonide.

2.3 Sex steroids

Sex steroids are steroid hormones which act by interacting with vertebrate androgen or estrogen receptors. Synthetic estrogens and progestins are used as methods of hormonal contraception. Ethinyl estradiol and levonorgestrel are forms of estrogen and progesterone, which are both female hormones involved in conception. Ethinyl estradiol and levonorgestrel are used together as an emergency contraceptive (EC) to prevent pregnancy after contraceptive failure or unprotected intercourse. Ethinyl estradiol and levonorgestrel prevent ovulation (the release of an egg from an ovary), disrupt fertilization (joining of the egg and sperm), and inhibit implantation (attachment of a fertilized egg to the uterus). Mechanism of action of the sex steroids is shown in the *figure no.03* [73].

The adverse events associated with the use of oral contraceptives (OC) are thromboembolic and thrombotic disorders, myocardial infarction, cerebral hemorrhage, cerebral thrombosis, carcinoma of the reproductive organs and breasts, hepatic neoplasia, ocular lesions, gall bladder disease, elevated blood pressure, headache and migraine. Many women discontinue using contraceptive pills primarily due to tolerability issues such as cycle control (bleeding irregularities), mood changes, nausea, bodyweight gain, breast tenderness, headaches, hypertension and fluid retention [12].

Drugs which cause the induction of metabolizing enzyme cytochrome P450 CYP3A4 enhance the clearance of oral contraceptive drugs. Cytochrome P450 enzyme inducers have been reported to increase the incidence of breakthrough bleeding and unwanted pregnancies in women using OC. Thus, it is very important to explore the potential interaction of new drug candidates with low dose OC during drug development process to ensure the optimum OC exposure to be maintained during concomitant therapy. To meet this need, a highly sensitive analytical method with a LLOQ in pg/mL level is essential [13,74].

Ethinyl estradiol (EE): Ethinyl estradiol (8R,9S,13S,14S,17R)-17-ethynyl-13-methyl-7,8,9,11,12,14,15,16-octahydro-6H-cyclopenta[a]phenanthrene-3,17-diol) is a synthetic estrogen has a molecular weight of 296.403, chemical formula $C_{20}H_{24}O_2$ and the following structure is presented in *figure no.04.*

Ethinyl estradiol developed in 1938 is one of the two steroid components, and an essential constituent of oral contraceptives (OC). It has high estrogenic potency when administered

orally, and is often used as the estrogenic component in oral contraceptives. In general, ethinylestradiol is used in combination with the progestrogen 19-norethindrone (NE) or levonorgestrel (LN) to prevent pregnancy in women [75.76].

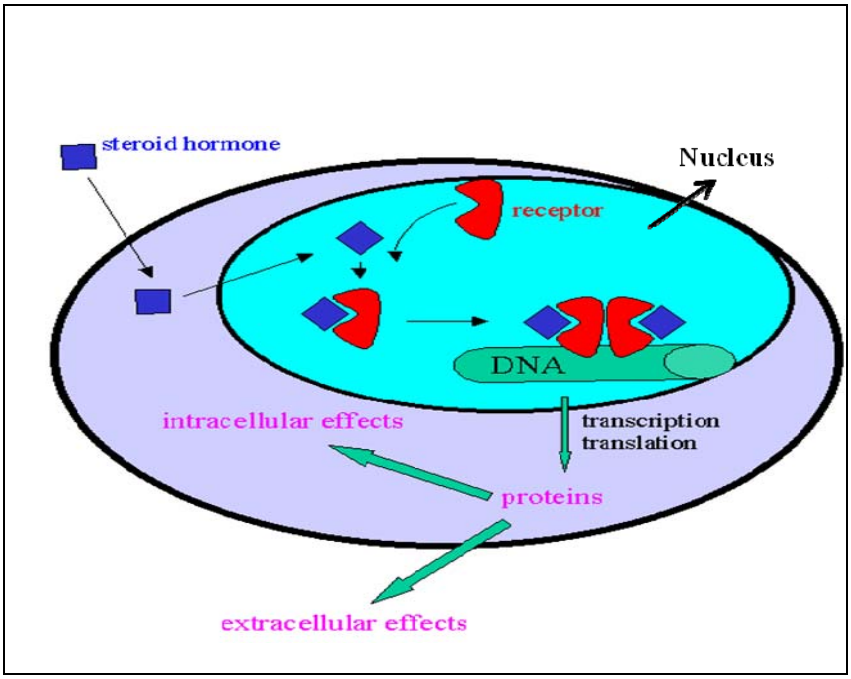

Figure No.03. Mechanism of action of Sex steroids

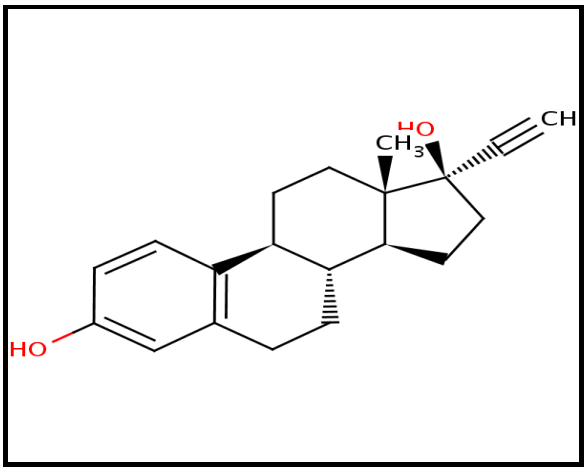

Figure No.04.Structure of Ethinyl estradiol

Ethinyl estradiol is primarily metabolized by 2-hydroxylation followed by glucuronide and sulfate conjugation. The CYP3A4 isoform of cytochrome P450 was reported to be the major form involved in the 2-hydroxylation of EE. With the introduction of low dose contributions

of OC, there has been a growing concern that their possible interaction with co-administered drugs might result in failure of contraception in women using OC. When a new OC formulation is developed, it is crucial to ensure optimum hormone exposure during concomitant therapy with other substances, while also guaranteeing the lowest dose to prevent pregnancy and avoid side effects. EE with LLOQ of 0.03ng/mL was achieved with LC-ESI-MS/MS[77]. To deal with these concerns, a highly sensitive analytical method with a low limit of quantification (LLOQ) in pg/mL level for EE is required to accurately measure OC concentrations in human plasma samples. [78]

For many years, the assay of EE in biological matrices was performed by radioimmunoassay methods.[79,80] These methods are sensitive, but are time consuming and prone to cross reactivity by endogenous steroids, co-administrated steroids and their metabolites. Therefore, liquid–liquid extraction (LLE) followed by HPLC had to be performed before RIA analyses. Using these rather tedious sample preparation procedures, RIA assays with a highly selective antiserum could typically achieve a lower limit of quantitation (LLOQ) of 2 pg/mL[81]. Methods using GC/MS typically involved an LLE or SPE extraction, derivatization and chemical ionization (CI) with selected ion monitoring (SIM) detection. However, the use of caustic reagent gas such as ammonia, the relatively long analysis time, and more importantly, the lack of derivatization information in the literature made GC/MS unsuitable for the high-throughput analysis of large number of samples.[82]

The electrospray-tandem mass spectrometry (ESI-MS–MS) in negative ionization mode for ethinylestradiol has become a dominant technique for its determination. Reported limits of detection varied from 0.08 to 10 pg/mL of ethinylestradiol depending on matrix composition, method of sample preparation and model of mass spectrometer used. Matejicek and Kuben reported a method based on a liquid chromatographic/iontrap mass spectrometric method for the quantification of ethinylestradiol in a mixture of many other estrogenic substances in biological materials. [83]

Liquid chromatography coupled with atmospheric pressure ionization/tandem mass spectrometry (LC-API-MS/MS) or atmospheric pressure photo spray ionization tandem mass spectrometry (LC-APPI-MS,MS) has been applied for the quantitative analysis of estrogens in environmental and biological samples because of its sensitivity and selectivity.[110,111,112] However, due to non-polar nature of the molecule, without derivatization the required

sensitivity is not reached. Chemical derivatization has been reported in literature to improve the electrospray sensitivity of analytes.[84] Anari et al., have reported LC-MS/MS method with dansyl chloride derivatization of EE in monkey plasma with a LLOQ of 5 pg/mL.[85] Sou et al. have reported a LC/MS-MS method for the determination of EE in human plasma with a LLOQ of 2.5 pg/mL for sample volume 1mL and analysis run time of 3.50 minutes.[86] Therefore liquid chromatography coupled to with tandem mass spectrometric detection is superior to immunoassay methods or GC-MS in terms of selectivity, sensitivity, simplicity and analytical throughput.

Levonorgestrel (LEV): Levonorgestrel [18,19-Dinorpregn-4-en-20-yn-3-one-13-ethyl-17-hydroxy-, (17α)- (-)-], is a synthetic progestogen, has a molecular weight of 312.45, chemical formula $C_{21}H_{28}O_2$. Structure is presented in the *figure no.05*

Levonorgestrel is used as an oral contraceptive since the mid 1960s as a progestin-only emergency contraceptive (EC) for women who had unprotected sexual intercourse to prevent pregnancy and, administered at lower doses either alone or in combination with an estrogen, as an oral contraceptive[87]. LEV possesses strong progestational and antiovulatory activities with no estrogenic effects. Changes in formulation have brought about a lowering the effective dose; thereby lowering clinical side effects. Monitoring of plasma levels of this drug is important to provide information for pharmacokinetic or bioavailability studies. Radioimmuno assay (RIA) methods have been used for determination of levonorgestrel during pharmacokinetic studies. [88] These methods are sensitive, but are expensive, time consuming, prone to cross reactivity by endogenous steroids, co-administrated steroids and their metabolites, hazardous due to radioactive labeling and non-specific.

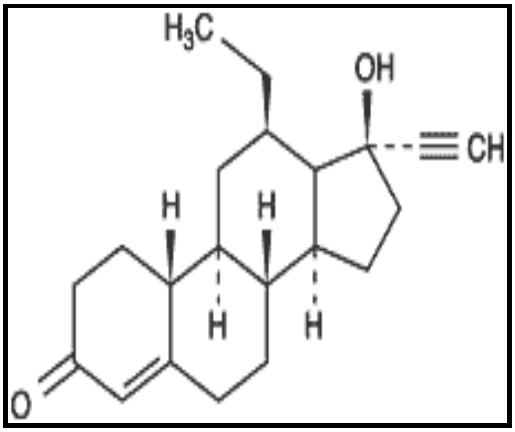

Figure No.05. Structure of Levonorgestrel

Reported HPLC methods are not sensitive enough to quantify levonorgestrel in pharmacokinetic analysis of low doses [89]. Gas chromatographic/mass spectrometric (GC/MS) methods typically employ some type of extraction (liquid–liquid or solid phase), and one or multiple steps of derivatization. [90] Theron et al. have reported an atmospheric pressure photospray ionization source to lower the background noise, but the sample preparation procedure required a freezing bath at -25°C to achieve layer separation which made the operation complicated. In addition, a large plasma volume (1 mL) was required to obtain a low LLOQ of 0.265 ng/mL.[91] LC–MS/MS has proved to be the preferred method for quantitative determinations due to rapid, highly selective and sensitive analysis. [92]

Mifepristone (MIF): Mifepristone is a substituted 19-nor steroid compound chemically designated as 11β-[*p*-(Dimethylamino) phenyl]-17β-hydroxy-17-(1-propynyl) estra-4,9-dien-3-one, with a molecular weight of 429.6. Its chemical formula is $C_{29}H_{35}NO_2$ and structural is presented in *figure no.06.*

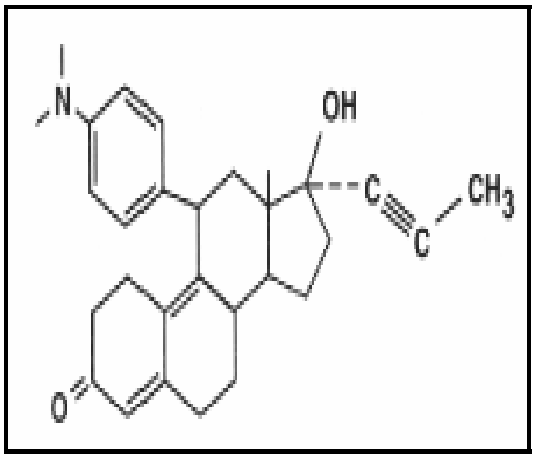

Figure No.06. Structure of Mifepristone

Mifepristone is a synthetic steroid used as an abortifacient in the first two months of pregnancy, and in smaller doses as an emergency contraceptive. It acts by blocking the hormone progesterone. The common side effects are nausea, vomiting, diarrhea, headache, dizziness, and fatigue. Bleeding and cramping are expected during this treatment [93,94]

Mifepristone has high affinity for the progesterone and glucocorticoid II (GRII) receptors and a low affinity for the androgen receptor. The dimethylaminophenyl side-chain on carbon 11 is important for the antiprogestogenic action of mifepristone [96]. A variety of methods for the determination of mifepristone in serum have been reported, mainly based on

radioimmunoassay (RIA) [97], radioreceptorassay (RRA) [98] and high-performance liquid chromatography (HPLC) [99,100] compared with the HPLC methods, the direct RIA and RRA methods have higher sensitivity. RIA and RRA methods are not specific for the determination of mifepristone and its metabolites because of the cross-reacting metabolites. These methods require use of radioactive materials, are expensive, time consuming and produce waste which is difficult to dispose. Stith and Hussian[99] have reported solid-phase extraction with HPLC-UV detection, with a linear range of 10–1000 ng/mL. Sensitivities of these methods were poor and operations were cumbersome.

Guo Z et al. have reported HPLC-UV detection method monitoring at wavelength of 302nm. The samples were extracted by solid-phase extraction and method was linear over a concentration range of 3-1000ng/mL with the run time of 15 minutes[101] . However when UV detection alone is used there is still potential for interference from co-eluting substances, in particular closely related metabolites which also absorb UV light at 302 nm. Reported liquid chromatography–tandem mass spectrometries (LC–MS/MS) are time consuming due to more analysis run time [102].

2.4 Chronopharmacology

Many studies on the individualization of pharmacotherapy have been carried out aiming at improvement of the pharmacotherapy. Chronopharmacokinetics deals with the study of the temporal changes in absorption, distribution, metabolism and elimination and thus takes into account the influence of time of administration on these different steps. The suprachiasmatic nuclei (SCN) of the anterior hypothalamus are the site of the circadian pacemaker in animals, which act like a multifunction timer to regulate homeostatic systems such as sleep and activity, hormone levels, appetite, and other functions of the body with 24 hr cycles[24] (*figure no.07*). The circadian clock is made up of three components: an input pathway adjusting the time, a central oscillator generating the circadian signal [103,104], and an output pathway manifesting itself in circadian physiology and behavior. The daily changes in light intensities are thought to be the major environmental cue involved in circadian entrainment. Light signals are perceived by photoreceptor cells in the retina and transmitted to neurons of the SCN via the retinohypothalamic tract. Environmental time cues, termed synchronizers or zeitgebers, the strongest being the daily light–dark cycle occurring in conjunction with the wake–sleep routine, set the inherited pacemaker circadian timekeeping systems to 24 hr each day.

The SCN of the anterior hypothalamus is the site of the circadian pacemaker in mammals. Clock genes, like Bmal 1, Per, Cry, are the genes that control the circadian rhythms in physiology and behavior. The effectiveness and toxicity of many drugs vary depending on dosing time associated with 24 hr rhythms of biochemical, physiological and behavioral processes under the control of circadian clock. The quantitative response (duration or intensity of the action) of an organism, as well as the qualitative response (i.e. inhibition or induction), varies with time of drug administration [105].

Chronopharmacology has been described in different areas such as anesthesiology, cardiology, oncology, endocrinology, gastroenterology, obstetrics, neurology, pneumology, psychiatry and rheumatology, in animals and humans. [105,106,107]

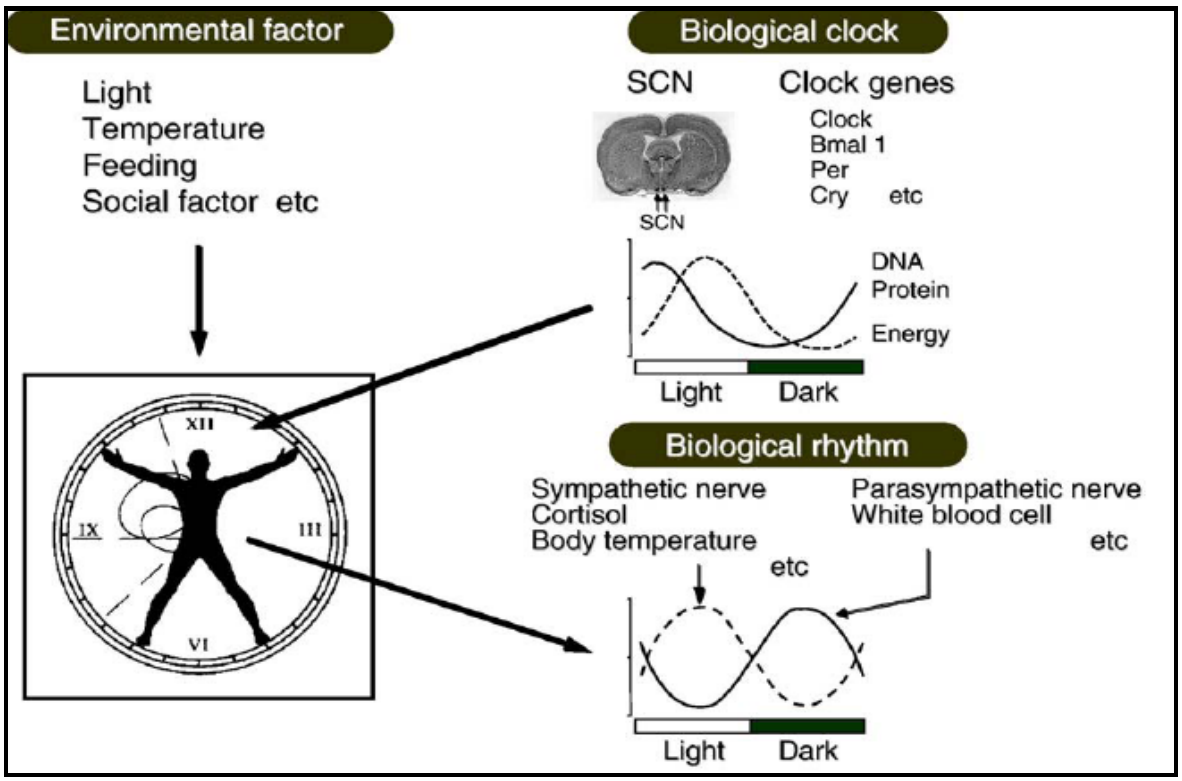

Figure No.07. Schematic diagram of the circadian system in mammals

The concept of periodic and predictable changes in drug effects can be understood by studying the chronokinetic for a drug (dosing time-dependent and predictable (rhythmic) changes in pharmacokinetic parameters C_{max}, t_{max}, AUC and $t_{1/2}$), the chronesthesy (rhythmic changes in susceptibility of the target biosystem to the drug) and the chronergy (the drug induced effect). The main aim of chronopharmacology refers to the use of a chronopharmacological approach

to clinical treatment so as to enhance both effectiveness and tolerance of a drug by determining the best biological time for its administration. Many studies have reported the influence of time of administration on acute toxicity of drugs [26,105]. Qualitative and quantitative responses to drugs in animals and humans have clearly demonstrated that all organisms are highly organized according to circadian rhythms.

Chronopharmacokinetic studies of cardiovascular drugs have shown that drugs such as nifedipine, oral nitrates and propranolol have a 2-fold higher C_{max} and shorter t_{max} after morning compared with evening time of administration. However, these kinetic variations were not detected when a sustained release dosage form (nifedipine and isosorbide mononitrate) was used. Since most of these drugs are lipophilic, the underlying mechanisms of their chronokinetic pattern involve a faster gastric emptying time and a higher gastrointestinal perfusion in the morning [107,108].

Mechanism of Chronopharmacokinetics

The data obtained from chronobiological studies have shown that, time-dependent changes in kinetics may result from circadian variations at each step of the fate of drugs in the organism (e.g., absorption, distribution, metabolism, and elimination) and vary along the 24 hrs scale including gastrointestinal, cardiovascular, hepatic and renal changes [109] as indicated in *figure-no.08.*

Circadian variations in drug absorption: The absorption of the drug after oral administration may be influenced by many factors, such as the physicochemical properties of the drug, the formulation of a drug, the area and the structure of the biomembrane, gastric emptying, pH and motility, and gastrointestinal blood flow. The influence of posture and the presence of food, composition of the food also affects bioavailability of the drug.[110] Gastric acid secretion and pH, motility, gastric emptying time, and gastrointestinal blood flow vary along the 24 hrs scale.[111,112]

Most of the lipophilic drugs seem to be absorbed faster when the drug is taken in the morning compared with the evening, but this has not been reported for highly water soluble drugs.

Absorption by other routes of administration may also be influenced by biological rhythms. For instance, in children, the skin penetration of an eutectic mixture of lidocaine (lignocaine) and prilocaine was reported to depend on the time of administration, with a higher rate of

penetration occurring in the evening[113]. Circadian variations have also been reported in the ocular absorption of topically applied timolol[109]. While most chronokinetic studies have been carried out with drugs administered orally, the persistence of significant chronokinetic changes with intravenous, compared with oral, administration suggests that the absorption process does not interfere with chronokinetic changes. Thus all factors including route of administration, feeding conditions, posture, and galenic formulation need to be controlled, taking into account the concerned biological rhythms.

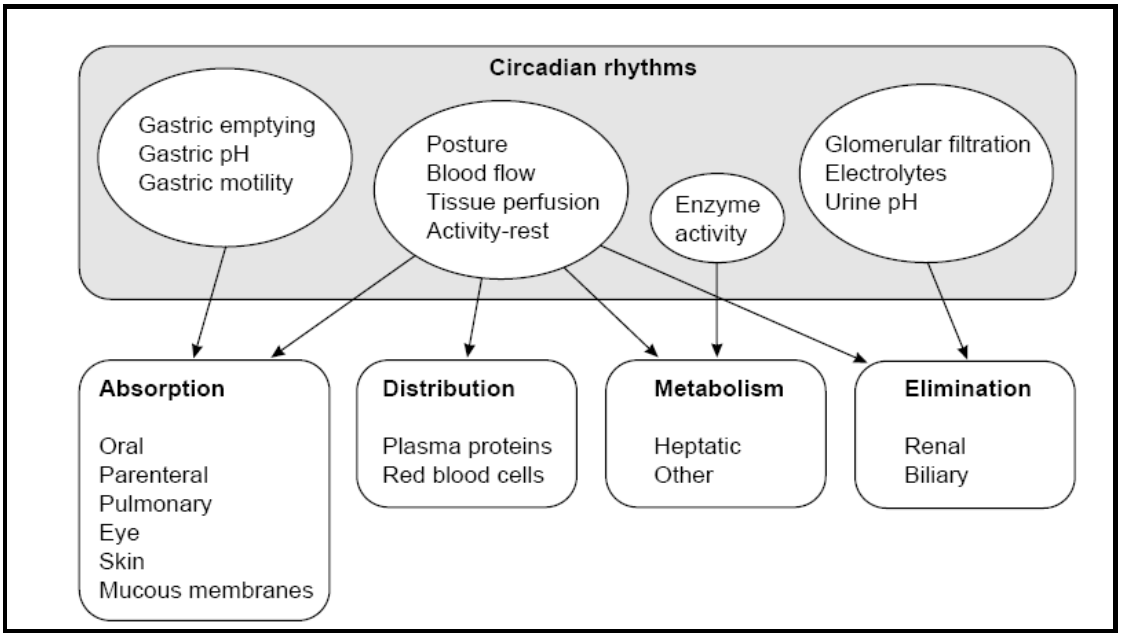

Figure No.08. Circadian rhythms of physiological variables involved in chronokinetic mechanism,

Circadian variations in drug distribution: Circadian changes in biological fluids and tissues have been documented to vary along the 24 hrs scale; such changes may obviously be implicated in drug distribution. Blood flow depends on several regulatory factors including sympathetic and parasympathetic systems, the activities of which are known to be circadian time-dependent with a predominant diurnal effect of the sympathetic system in man. Thus, diurnal increase and nocturnal decrease of blood flow and distribution in tissue may explain a possible difference in drug distribution according to time of administration. Circadian

variations in the drug protein binding of acidic and basic drugs have been reported both in human and animal studies. Free plasma concentrations have been documented for anticonvulsant drugs, such as carbamazepine, diazepam, phenytoin and valproic acid, and for antineoplastic drugs such as cisplatin. These changes are usually reported to be dependent on temporal variations in plasma protein levels, which are circadian time dependent [25].

These changes may also depend on factors such as temperature, pH, and physicochemical properties of the concerned drug which may possibly be subject to temporal variations. Ando et al. have reported variations of P-glycoprotein (Pgp), a multidrug transporter which contributes to renal, biliary, and intestinal elimination of drugs [114]. The drugs that are characterized by a high protein binding (>80%) and a small apparent volume of distribution (Vd) may have clinical implications in plasma drug binding based on temporal variations, but the clinical consequences of circadian variations in plasma protein levels have not yet been demonstrated.

The time-dependency of the passage of drugs into red blood cells also provides a strong argument for the existence of temporal variations in the passage of drugs through biological membranes, for which red blood cells are often used as a model. Circadian time-dependent changes in the passage of drugs such as local anesthetics (Lidocaine, Bupivacaine, Etidocaine, and Mepivacaine), Indomethacine, Theophylline, etc., into red blood cells have been reported [25].

Circadian variations in drug metabolism: Drug metabolism is generally assumed to depend on liver enzyme activity and/or hepatic blood flow: both have been shown to be circadian time-dependent [115]. Circadian variations in enzyme activity were documented in other tissues like liver, kidney and brain [116]. For drugs with a high extraction ratio, hepatic metabolism depends on hepatic blood flow. Circadian variations in hepatic blood flow induce changes in liver perfusion and, thus, temporal variations in the clearance of such drugs. Studies have shown that various oxidative reactions catalyzed by the hepatic microsomal monooxygenases systems have temporal variations, thus changes with oxidation, reduction, hydrolysis, and conjugation were shown to be circadian time-dependent. Ohno et al. have reported circadian variations in the urinary 6 beta-hydrocortisol to cortisol ratio in man; indicative of temporal variations in cytochrome P-450 3A activity [117]. As far as metabolic phenotype is concerned, Shaw et al. have shown the effect of diurnal variation on debrisoquine metabolic phenotyping,

with the slowest rate of metabolism occurring during the daytime [118].

Circadian variations in drug excretion: Most drugs are eliminated via the kidneys. Many renal physiological functions such as glomerular filtration, renal blood flow, urinary pH and tubular resorption have all been shown to be circadian time-dependent, with higher values during the daytime in humans [117]. These rhythmic variations in renal functions may have consequences on drug urinary excretion of hydrophilic drugs (mainly excreted unchanged by the kidneys). Renal elimination depends partially on the ionisation of drugs, and thus may be modified by temporal changes in urinary pH. This has been described for acidic drugs such as sodium salicylate and sulfamethoxazole [119] which are excreted more quickly after evening than morning administration.

Need of the Chronopharmacokinetic study: Chronopharmacokinetics is not taken into account during the drug development process due to lack of sensitive methodologies required, the clinical relevance of chronopharmacokinetics, the cost involved in the studies or their poor benefit/cost ratio,. Chronobiological circadian variations have been determined for many physiological functions that may affect drug disposition and pharmacological response. Chronopharmacokinetic study is of clinical relevance and instances in which chronopharmacokinetic studies are desirable include a) time dependent variations in pharmacokinetics on drug effects is observed, b) the drug has a narrow therapeutic range, or its plasma levels need to be monitored in order to adapt better dosing, c) when the symptoms of a disease are clearly circadian phase dependent (e.g. nocturnal asthma, angina pectoris, myocardial infarction, ulcer disease) and d) when the drug is characterized by a high inter and intra-subject variability [27].

Bleyzac et al. have reported a significant decrease in the clearance of amikacin after administeration in the evening possibly due to diurnal variations in glomerular filtration rate [160]. Satoh et al. reported circadian variations of mycophenolate kinetics, daytime Cmax and AUC were higher than the night time values, while t_{max} was shorter after the morning dose compared to the evening dosing. These changes are clinically relevant since a lower mycophenolate exposition (AUC) is associated with the occurrence of acute rejection in the early stages after renal transplantation [120].

Chronopharmacology examines the influence of the time of drug administration on the drug and body response according to the temporal variations. Thus the quantitative response

(duration or intensity of the action) of an organism, as well as the qualitative response (i.e. increase or decrease of its effect), varies with time of administration. Moreover, the different steps in pharmacokinetics, drug absorption, distribution, metabolism and elimination, are influenced by different physiological functions that may vary with the time of day. Thus, pharmacokinetic parameters like, peak drug plasma concentration (C_{max}), time to reach C_{max} (t_{max}), AUC, elimination half-life ($t_{1/2}$) and clearance are considered to be circadian time-dependent [120]. Neglecting the possible influence of biological rhythms on drug kinetics contributes to enhanced variability. The main aim of chronopharmacokinetic studies is to control the time of drug administration, which can be responsible for variations of drug kinetics but may also explain chronopharmacological effects observed with drugs.

Chapter-3

AIMS AND OBJECTIVES

Bioanalytical methods are used for the quantitation of drugs and their metabolites in biological matrices. The present discovery and drug development scenario calls for highly sensitive and selective methods to quantify drugs in matrices such as blood, plasma, serum or urine. Chromatographic methods (high-performance liquid chromatography [HPLC] or gas chromatography [GC] have been widely used for the analysis of small molecules, while liquid chromatography coupled to triple quadrupole mass spectrometry (LC-MS/MS) is the single most commonly used technology for bioanalysis. After developing a method with desired attributes, the method is validated to establish that it will continue to provide accurate, precise, and reproducible data during the complete duration of the study. Method validation is a process that demonstrates that the method will successfully meet or exceed the minimum standards recommended in the Food and Drug Administration (FDA) Guidance, for accuracy, precision, selectivity, sensitivity, reproducibility, and stability. Validation is performed using a control matrix spiked with the analyte/s to be quantified.

High-performance liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) due to its high degree of sensitivity and selectivity represents the wide use for quantitative bioanalysis of small molecules. It can be routinely employed in a clinical environment and is attractive because of simplicity of sample processing and high throughput. Mass spectrometry can be regarded as a complementary technique having technical advantages over immunoassays and gas chromatography for the determination of steroids in biological matrices. The ability of mass spectrometry methods to detect low levels of steroids enhances their clinical use particularly at extremely low concentration levels.

Corticosteroids are used to treat various inflammatory and immunology mediated diseases. Prednisone, Prednisolone, Dexamethasone, Betamethasone, Fluticasone propionate, Triamcinolone acetonide and Budesonide are among the most frequently used corticosteroids and their determination in biological fluids is necessary. Corticosteroids and sex steroids can be estimated by immunoassay and Gas chromatography–mass spectrometry (GC–MS) methods. Immunoassays suffer from lack of selectivity and necessitates the use of different types of kits to cover the wide range of corticosteroids. Gas chromatography–mass spectrometry (GC-MS) methods suffer from drawbacks like long analysis time and time consuming derivatization. Prolonged or systemic use of corticosteroids leads to significant side effects. Low-dose corticosteroids may provide a favorable benefit/risk ratio for the

therapeutic applications which requires quantification of drugs at very low concentrations in plasma. Developing the methods with high sensitivity and selectivity for corticosteroid pharmacokinetics (PK) is a challenging task; as a result LC–MS methods are now frequently applied for the determination of steroids.

The main problem associated with LC-MS/MS during the determination of analytes of interest in plasma is matrix effect. Molecules originating from the sample matrix that coelute with the compound(s) of interest can interfere with the ionization often resulting in false MS signals and responses.

The present work aims at the development of selective, sensitive and rapid LCMS-MS method for determination of steroids in human plasma using selective reaction monitoring (SRM) mode. Efforts are made to eliminate co-eluting components of plasma, since their presence will reduce method sensitivity.

There are numerous investigations demonstrating that pharmacokinetics and drug effects can depend on the time of drug administration and on biological rhythms of physiological functions. Studies in animals and humans have provided evidence for regular and predictable circadian and circannual variations in pharmacodynamics and pharmacokinetics. Circadian (about 24 hr) rhythms exist at all levels of organization. There are circadian rhythms in sleep and activity patterns, in plasma cortisol levels (which reaches its maximal levels early in the morning in healthy humans) and in body temperature. These rhythms are driven by biological clocks and are endogenous in origin. A consequence of these rhythmic variations in biology is that both drug disposition and effects vary as a function of the time of drug administration. The temporal changes in drug effects include variations both in the desired (chronoeffectiveness) and undesired (chronotoxicity) effects. When considering circadian effect of the drugs, it is obvious that the fate of a drug may vary according to the time of its administration.

However, despite numerous experimental and clinical chronopharmcokinetic studies, the time of administration of a drug still remains a neglected factor in many kinetic studies; the reasons for which may be,

(a) Non availability of methodological aspects in order to correct the dose or therapy with circadian rhythm.

(b) Ignorance or denial of the relevance of chronobiological data.

There is no doubt that the pharmacokinetics can be significantly influenced by time of administration. Time of day has to be regarded as an additional variable to influence kinetics of a drug. In present research work attempt is made to find circadian variation in the pharmacokinetic parameters of selected corticosteroids and sex steroids as application to developed methods.

AIM OF THE WORK

The present research work, aims at developing and validating the bioanalytical method for the selected synthetic corticosteroids and sex steroids and its application to chronopharmacokinetics.

OBJECTIVES

1. Bioanalytical method development and validation for simultaneous estimation of selected corticosteroids like Budesonide, Fluticasone, Prednisone, Prednisolone, Dexamethasone and Triamcinolone acetonide in human plasma as per the USFDA guidelines.
2. Bioanalytical method development and validation of Ethinyl estradiol in human plasma as per the USFDA guidelines.
3. Bioanalytical method development and validation of Levonorgestrel in human plasma as per the USFDA guidelines.
4. Bioanalytical method development and validation of Mifepristone in human plasma as per the USFDA guidelines.
5. Application of developed and validated bioanalytical methods to chronopharmacokinetic study of selected molecules like Prednisolone, Ethinyl estradiol, Levonorgestrel and interpretation of the data.

Chapter-4

BIOANALYTICAL METHOD FOR SIMULTANEOUS DETERMINATION OF BUDESONIDE, FLUTICASONE PROPIONATE, PREDNISOLONE, PREDNISONE, DEXAMETHASONE AND TRIAMCINOLONE ACETONIDE IN HUMAN PLASMA

Corticosteroids are used for the treatment of a wide range of diseases and are among the most frequently prescribed drugs for anti-inflammatory and immunomodulatory effects. Measurement of plasma concentrations of synthetic corticosteroids is useful for assessing suspected iatrogenic hypothalamic-pituitary-adrenal axis suppression and Cushing's syndrome.

Establishing a definitive diagnosis of systemic synthetic corticosteroid effects and accurate monitoring requires measurement of actual synthetic steroid concentrations in biological fluids. Such assays are generally not available in hospital, and research laboratories, and if existing they may allow measurement of only a single synthetic corticosteroid. Methods have been reported specifically to analyze a single or only a few synthetic corticosteroids and their metabolites. The doses of Budesonide and Fluticasone propionate delivered to the lungs are very small and result in very low plasma concentrations; therefore highly sensitive methods are required for their estimation. Prednisone, Prednisolone and Dexamethasone are of similar structure with endogenous cortisol, cortisone and hydrocortisone, therefore the method needs to be highly specific. Most of the reported analytical methods are of inadequate sensitivity and require long analysis run time. Therefore there is a need of highly sensitive, selective, rapid and rugged bioanalytical method for the simultaneous determination of corticosteroids in biological fluids.

4.1 Reference/Working Standards

The reference/working standards used were from Manipal acuNova reference standards Bank.

Standards	**Suppliers/Manufacturers**
Prednisolone	Sigma,USA
Prednisone	Sigma,USA
Triamcinolone	Sigma,USA
Budesonide	Sun Pharmaceuticals, India*
Dexamethasone	Sigma,USA
Fluticasone propionate	Sun Pharmaceuticals, India*
Imipramine Hydrochloride	Sigma,USA

*obtained as gift samples from Sun Pharma, Baroda, India

4.2 Reagents and chemicals

All the chemicals and reagents used were of standard grade and the details of which are as given under.

Chemicals	Grade	Manufacturer
Methanol	Super gradient	Merck, Germany/Labscan, Thailand JT Baker, USA.
Ammonium Acetate	Fractopur/AR grade	Merck, Germany/ S.d.Fine-chem Ltd., Mumbai, India
Ammonium formate	GR grade	Merck Specialists Pvt Limited, Mumbai, India
Water	HPLC/Milli-Q	Qualigens, India/Millipore, USA
Tertiary-butyl methyl ether	HPLC	Lab Scan, Thailand/Merck India
Formic acid	Analytical	Merck, Germany
Acetonitrile	Super gradient	Merck, Germany/Labscan, Thailand JT Baker, USA.
n-Hexane	GR grade	Merck Specialists Pvt Limited, Mumbai, India
Ethyl acetate	HPLC	Lab Scan, Thailand/Merck India
Dichloromethane	HPLC	Merck, Germany
Perchloric acid	Analytical	Merck, Germany
Hydrochloric acid	Analytical	Merck, Germany

4.3 Blank plasma

Harvested K_2 EDTA blank plasma for method development and validation was obtained Navajeevan blood bank, Hyderabad, India and Blood Bank, Kasturba Hospital, Manipal, India.

4.4 Instruments

The instruments used for the method development and validation are listed below,

Instrument Name	Make/Model	Manufacturer
Auto sampler	Surveyor	Thermo Finnigan Inc. USA
Analytical balance	Sartorius (CP225D)	Sartorius Mechatronics, India
Analytical Columns	Genesis,C-18, 100×4.6mm, 4µ	Grace, USA
	Chromolith,C-18, 50×4.6mm, 3µ	Merck, Germany
	Ace, C-18, 35×4.6mm, 3µ	ACT, Scotland
	Betasil 50×4.6mm, 5µ, C-18	Thermo Scientific
	Hypersil gold, 50×4.6mm,C18, 5µ,	Thermo scientific
	X- terra,RP 18, 50×4.6mm,C18, 5µ	Waters
Column oven	Surveyor	Thermo Finnigan Inc. USA
Centrifuge	Heraeus	Thermo Electron Corporation, USA
Deep Freezer	995-Forma -86C	Thermo Electron Corporation
Hot air oven	JRIC 7/A	Osworld equipment's, Mumbai
HLB cartridges	Oasis	Waters, USA
Micro balance	Sartorius (SE2)	Sartorius Mechatronics, India
Micropipette	Eppendorf/Brand	Eppendorf/Brand, Germany
MS Detector	TSQ Discovery Max	Thermo Finnigan Inc. USA
Multipulse Vortexer	Glass-Col	Terre Haute, USA
Nitrogen Evaporator	TurboVap LV	Caliper Life Science
Solvent delivery module	Surveyor	Thermo Finnigan Inc. USA
Solid phase extraction unit	Ezypress	Orochem, India
Ultrasonic bath	Spectralab	Spectralab instruments Pvt ltd, Thane, Mumbai
Vortexer	Spinix	Spinix, India
Water purification system	Milli-Q	Millipore, USA.

4.5 Method development procedure and results

4.5.1 Scanning of analytes and optimizing MS/MS detector parameters

Method development involves scanning of the analyte solutions to find the parent ion and its respective fragment ions. For this purpose 100ng/mL solution of Budesonide (BUD), Fluticasone propionate (FLU), Prednisolone (PRE), Prednisone (PRD), Dexamethasone (DEX) and Triamcinolone acetonide (TRI) and Imipramine (IMI) used as internal standard were prepared in methanol. Solution of each analyte was injected separately using the syringe

pump and scanned for the parent mass of the analyte. Scanning was carried out in full scan mode to quantify the analytes of interest. Mass spectra of BUD, FLU, PRE, PRD, DEX and TRI and IMI were recorded by scanning in the range of 50 to 600 m/z. Once the parent ion was obtained it was further scanned for product ions using MS/MS mode. The fragment ion having higher intensity was selected for selective reaction monitoring (SRM). On optimization of the chromatographic conditions, each analyte was tuned manually in SRM mode with the mobile phase by using "T" which connects LC pump and syringe pump to the detector in order to optimize tube lens offset voltage, collision gas pressure, collision energy, sheath gas pressure, auxiliary gas pressure and capillary temperature to achieve maximum response.

The main objective of this work was to develop and validate a simple, rapid and sensitive method for the simultaneous determination of BUD, FLU, PRE, PRD, DEX and TRI in human plasma suitable for pharmacokinetic sample analysis in clinical studies. The basic principle of MS is the production of ions which are subsequently separated according to their mass-to-charge ratio (m/z) and detected. Argon gas was used as collision gas, nitrogen as sheath gas and the resolution was set to unit mass.

For optimizing instrument settings for the detection and fragmentation, 100 ng/mL solution of each analyte was directly infused into the mass spectrophotometer source. Selection of predominant and stable product ions for each compound is very critical in mass tuning to obtain reproducible and enhanced mass spectrometric selectivity. The relative abundance for all the molecules was found to be better when scanned in positive ion mode compared with the negative mode because of better electrospray ionization (ESI) of positively charged steroids. APCI did not show overall advantages over ESI during simultaneous determination of all the analytes The LC-MS/MS method using the SRM comprising the precursor and product ions was used for the detection of analytes.

The daughter ions (Q3) of all of the steroids obtained during collision of the precursor ions (Q1) in tandem MS are listed in *table No.01*. The results of mass scan are represented in *figures no: 09(a),(b),(c),(d),(e,(f) and (g))* for BUD, FLU, PRE, PRD, DEX, TRI and IMI respectively.

Table No. 01: SRM ion transitions for synthetic corticosteroids

Sl. No.	**Molecule name**	**Parent ion (Q1)**	**Product Ion (Q2)**	**CE** (v)	**Q1PW**	**Q3PW**	**Tube Lens**
1	Budesonide	431.20	413.13	10	0.70	0.70	100
2	Fluticasone propionate	501.20	274.80	25	0.70	0.70	100
3	Prednisolone	361.20	343.20	10	0.70	0.70	100
4	Prednisone	359.10	341.18	10	0.70	0.70	100
5	Dexamethasone	393.10	373.11	9	0.70	0.70	100
6	Triamcinolone acetonide	435.00	415.15	10	0.70	0.70	100
7	Imipramine hydrochloride	281.20	86.19	21	0.70	0.70	90

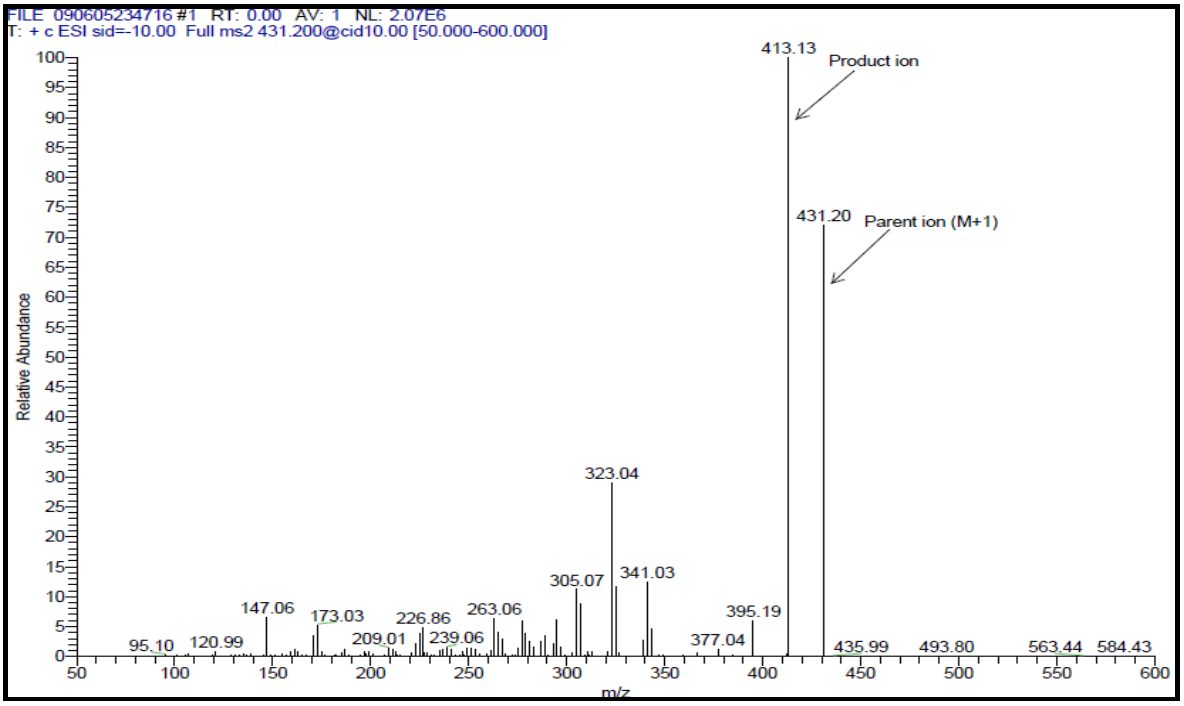

Figure No: 09(a). Parent and product scan analysis of Budesonide

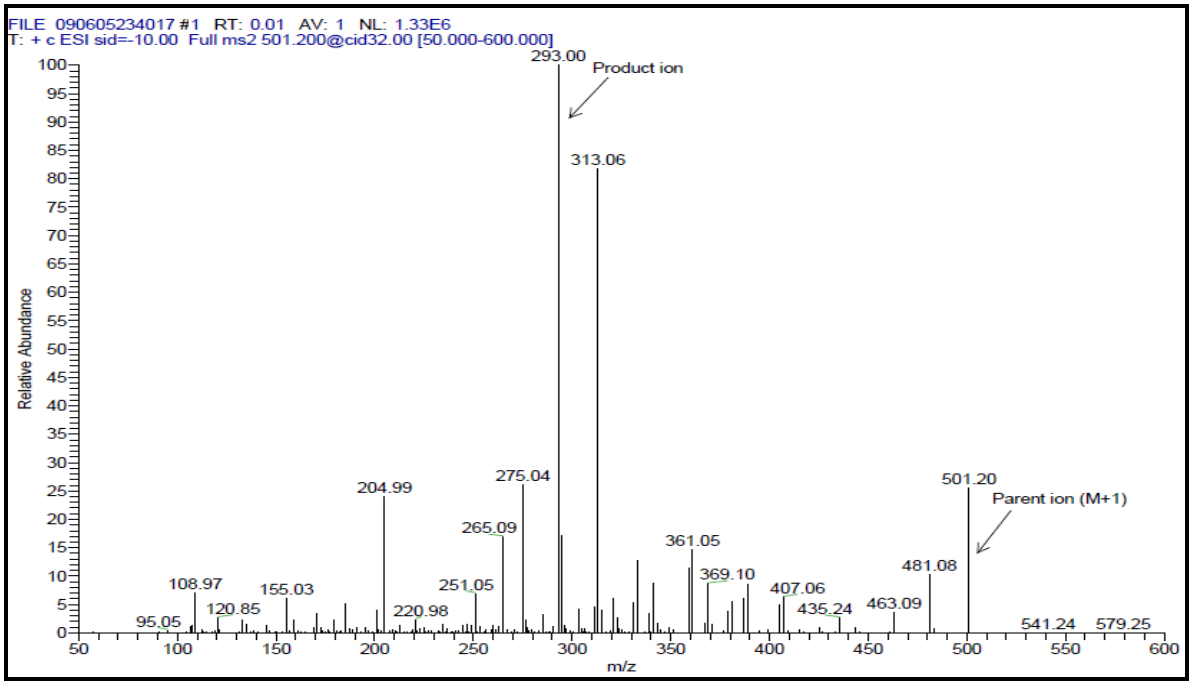

Figure No: 09(b). Parent and product scan analysis of Fluticasone propionate

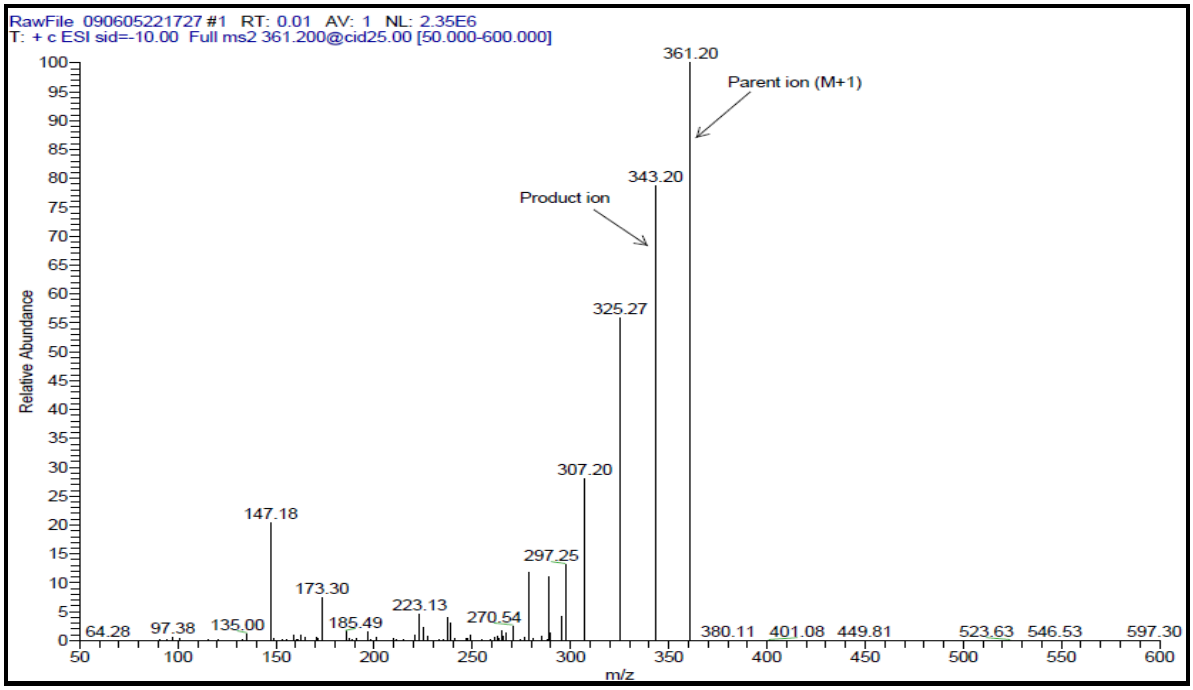

Figure No.09(c). Parent and product scan analysis of Prednisolone

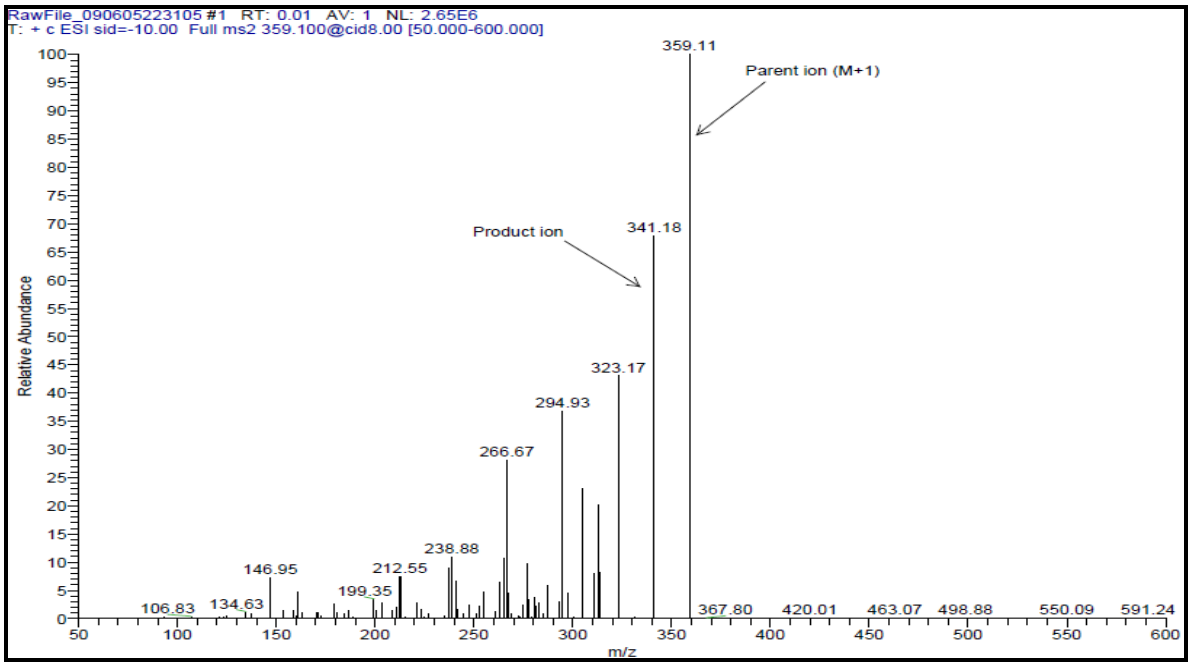

Figure No: 09(d). Parent and product scan analysis of Prednisone

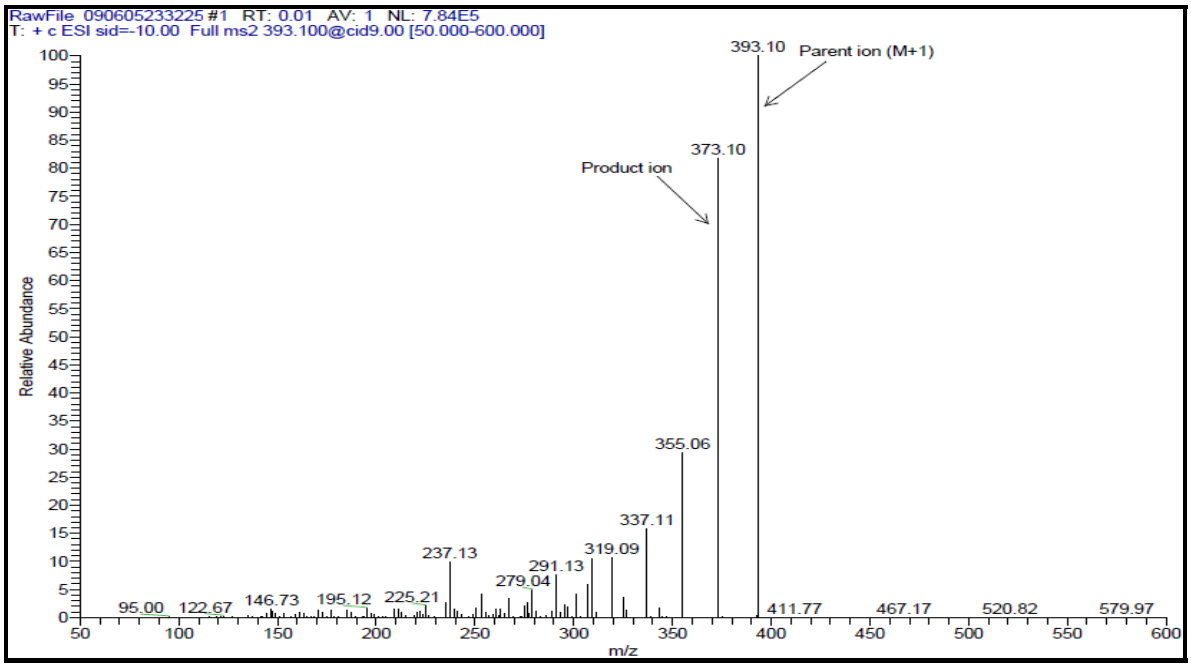

Figure No: 09(e). Parent and product scan analysis of Dexamethasone

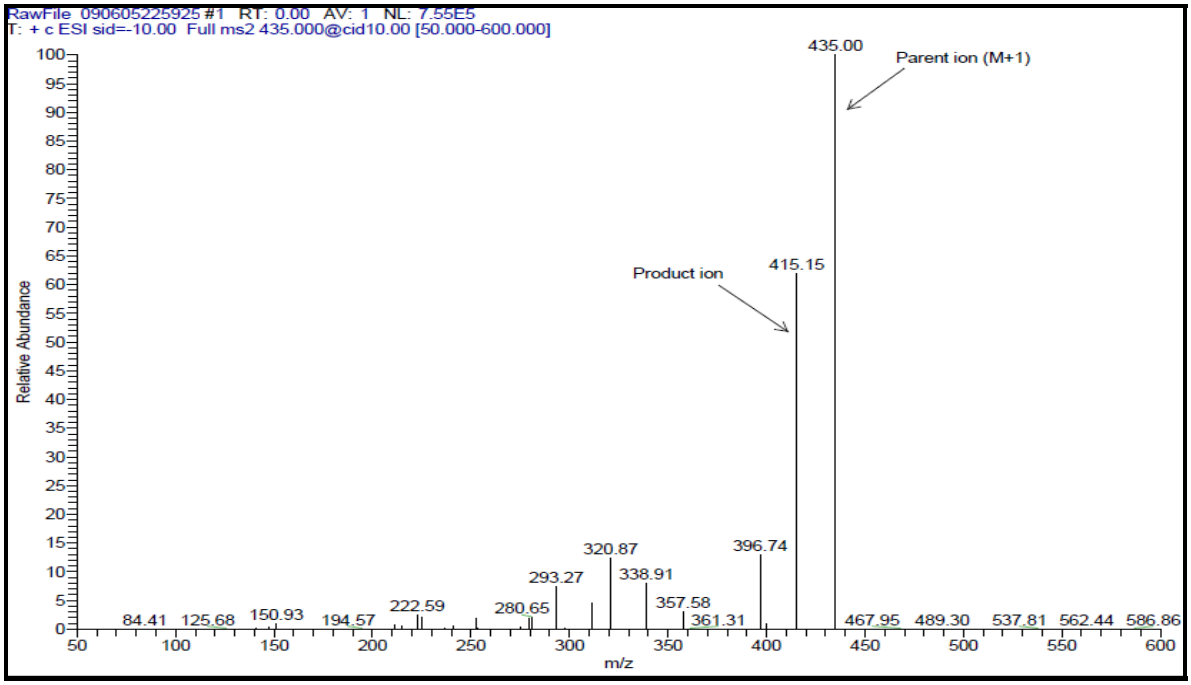

Figure No: 09(f). Parent and product scan analysis of Triamcinolone acetonide

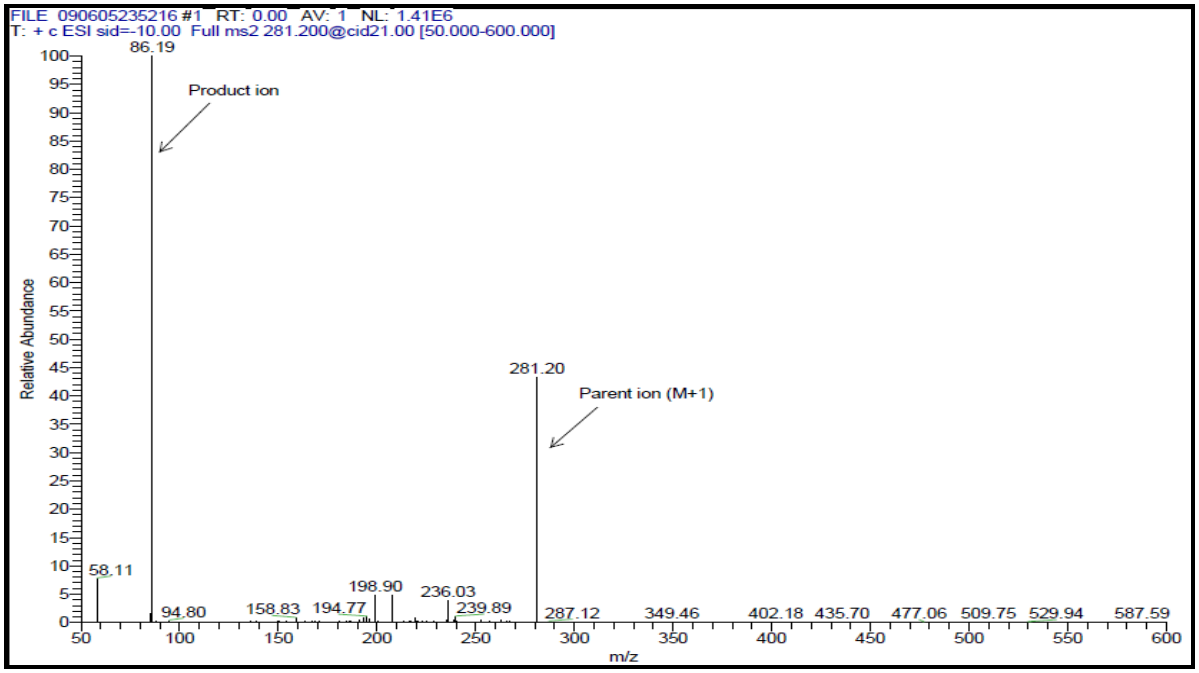

Figure No: 09(g). Parent and product scan analysis of Imipramine

4.5.2 Preparation of analyte main stock solutions

Main stock solution of BUD, FLU, PRE, PRD, DEX, TRI and IMI (1.000mg/mL) were prepared by weighing each of the reference/working standard equivalent to 10.000 mg into separate 10.000mL volumetric flasks, dissolved with 5.000 mL of methanol and diluted up to the mark with methanol.

4.5.3 Preparation of intermediate stock solutions

a) BUD and FLU intermediate stock solutions (50.000ng/mL) were prepared by transferring 0.025 mL of main stock solution (1.00mg/mL) into separate 50.000 mL volumetric flasks and the volumes were made up to 50.00mL with methanol: water (50:50%v/v). From this solution 1.000mL was transferred in to separate 10.00mL volumetric flasks and volumes were made up to the mark with methanol: water (50:50%v/v).

b) Preparation of Triamcinolone acetonide intermediate stock solution (10.000 µg/mL and 50.000 µg/mL)

0.100mL and 0.500mL Triamcinolone acetonide main stock solution(1.00mg/mL) was pipetted into separate 10.000mL volumetric flasks and the volume was made up to mark with methanol: water (50:50%v/v).

c) Preparation of mixed intermediate stock solution (Dexamethasone-12.500µg/mL, Prednisolone-50.000µg/mL, Prednisone-25.000µg/mL)

0.125mL of Dexamethasone (1.00mg/mL), 0.500mL of Prednisolone (1.00mg/mL), 0.250mL of Prednisone (1.000mg/mL) main stock solutions were pipetted in to a 10.000mL volumetric flask and the volume was made up to the mark with methanol: water (50:50%v/v).

d) Preparation of Imipramine internal standard working solution (1.000µg/mL).

0.010mL of Imipramine main stock solution was transferred into a 10.00mL volumetric flask and the volume was made up to the mark with methanol: water (50:50%v/v).

4.5.4 Preparation of spiking stock solutions

4.5.5.1 Preparation of calibration standards spiking stock

Volume of intermediate stock taken for the preparation of calibration standards was as per the table given below,

Calibration standard	Volume of intermediate stock solution taken(for 10.000 mL of spiking stock)			
	Mixed intermediate for DEX, PRE and PRD	BUD	FLU	TRI
STD-1	0.002	0.010	0.020	0.002*
STD-2	0.004	0.020	0.040	0.004*
STD-3	0.100	0.100	0.100	0.100
STD-4	0.400	0.200	0.200	0.200
STD-5	0.600	0.400	0.400	0.400
STD-6	0.800	0.800	0.800	0.800
STD-7	1.200	1.200	1.200	1.200
STD-8	1.600	1.600	1.600	1.600
STD-9	2.000	2.000	2.000	2.000

*For Triamcinolone acetonide STD-1 and STD-2 preparation10.000µg/mL intermediate stock solution was taken and for STD-3 to STD-9 50.000µg/mL intermediate stock solution was taken.

As per the above table the intermediate stock solutions were transferred in to a 10.000mL volumetric flasks and volume was made up to the mark with methanol: water (50:50%v/v). This resulted in the spiking stock concentrations as mentioned below,

Calibration standards	Spiking stock concentration					
	PRE (ng/mL)	PRD (ng/mL)	DEX (ng/mL)	BUD (pg/mL)	TRI (ng/mL)	FLU (pg/mL)
STD-1	20.00	10.000	5.00	200.00	2.00	200.00
STD-2	40.00	20.00	10.00	400.00	4.00	400.00
STD-3	1000.00	500.00	250.00	2000.00	1000.00	1000.00
STD-4	2000.00	2000.00	500.00	4000.00	2000.00	2000.00
STD-5	4000.00	3000.00	1000.00	8000.00	4000.00	4000.00
STD-6	8000.00	4000.00	2000.00	16000.00	8000.00	8000.00
STD-7	12000.00	6000.00	3000.00	24000.00	12000.00	12000.00
STD-8	16000.00	8000.00	4000.00	32000.00	16000.00	16000.00
STD-9	20000.00	10000.00	5000.00	40000.00	20000.00	20000.00

0.025 ml of the respective spiking stock solution was spiked in 0.475 ml of blank plasma, resulting in plasma concentrations for each drug as depicted in the table.

Calibration standards	Plasma concentration*					
	PRE (ng/mL)	PRD (ng/mL)	DEX (ng/mL)	BUD (pg/mL)	TRI (ng/mL)	FLU (pg/mL)
STD-1	1.00	0.50	0.25	10.00	0.10	10.00
STD-2	2.00	1.00	0.50	20.00	0.20	20.00
STD-3	50.00	25.00	12.50	100.00	50.00	50.00
STD-4	100.00	50.00	25.00	200.00	100.00	100.00
STD-5	200.00	100.00	50.00	400.00	200.00	200.00
STD-6	400.00	200.00	100.00	800.00	400.00	400.00
STD-7	600.00	300.00	150.00	1200.00	600.00	600.00
STD-8	800.00	400.00	200.00	1600.00	800.00	800.00
STD-9	1000.00	500.00	250.00	2000.00	1000.00	1000.00

*Spiking volume 5%v/v.

4.5.5.2 Preparation of Quality control samples spiking stock

Volume of intermediate stock taken for the preparation of quality control samples were as per the table given below,

Quality control	Volume of intermediate stock solution taken(mL)			
	Mixed intermediate for DEX, PRE and PRD	BUD	FLU	TRI
LLOQC	0.02	0.01	0.02	0.002*
LQC	0.06	0.03	0.06	0.006*
MQC	1.00	1.00	1.00	1.00
HQC	1.80	1.80	1.80	1.80

*For Triamcinolone acetonide LLOQC and LQC 10.000µg/mL intermediate was taken for and for MQC and HQC spiking stock solutions 50. 000µg/mL was taken.

As per the above table the volumes were transferred in to a 10.000mL volumetric flasks and volume was made up to the mark with methanol: water (50:50%v/v). This resulted in the spiking stock concentrations as mentioned below,

Quality control	Spiking stock concentration					
	PRE (ng/mL)	PRD (ng/mL)	DEX (ng/mL)	BUD (pg/mL)	TRI (ng/mL)	FLU (pg/mL)
LLOQC	20.00	10.00	5.00	200.00	2.00	200.00
LQC	60.00	30.00	15.00	600.00	6.00	600.00
MQC	10000.00	5000.00	2500.00	20000.00	10000.00	10000.00
HQC	18000.00	9000.00	4500.00	36000.00	18000.00	18000. 00

0.025 ml of the respective spiking stock solution was spiked in 0.475 ml of blank plasma, resulting in plasma concentrations for each drug as depicted in the table.

Quality control	Plasma concentration*					
	PRE (ng/mL)	**PRD (ng/mL)**	**DEX (ng/mL)**	**BUD (pg/mL)**	**TRI (ng/mL)**	**FLU (pg/mL)**
LLOQC	1.00	0.50	0.10	10.00	0.10	10.00
LQC	3.00	1.50	0.30	30.00	0.30	30.00
MQC	500.00	250.00	500.00	1000.00	500.00	500.00
HQC	900.00	450.00	900.00	1800.00	900.00	900.000

*Spiking volume 5%v/v.

4.5.5 Preparation of solutions and buffers

4.5.5.1 Preparation of solutions and buffers

Weighed amount of the salt was transferred in to a glass beaker and 500.000mL of milli-Q/HPLC water was added and sonicated to mix well. The solution was then filtered through 0.22µm membrane filter. Different strengths of the buffer were prepared by weighing the salts as shown below.

Sl. No.	strength of the buffer	Name of the salt	Weight taken(mg)
1	2mM	Ammonium acetate	77.08
2	5mM	Ammonium acetate	192.70
3	10mM	Ammonium acetate	385.40
4	5mM	Ammonium formate	157.65
5	10mM	Ammonium formate	315.15

4.5.5.2 Preparation of 0.1%v/v formic acid in water

0.100mL of formic acid was accurately transferred in to a 100.00ml volumetric flask and the volume was made up to the mark with HPLC water and sonicated to mix well.

4.5.5.3 Preparation of reconstitution solution ((Methanol: 5mM ammonium acetate (80:20% v/v)).

400.000mL of the methanol and 100.00mL of 5mM ammonium acetate was transferred into a glass bottle and sonicated to mix well.

4.5.5.4 Preparation of the diluent: 50% v/v methanol in water was prepared in a glass bottle with HPLC water and sonicated to mix well

4.5.6 Optimizing chromatographic conditions

4.5.6.1 Effect of pH, stationary phases, solvent strength and flow rate.

The standard solution was chromatographed for about 3-10 min using organic solvents such as methanol and acetonitrile and aqueous buffers such as water, ammonium formate, ammonium acetate and 0.1% formic acid. Effect of ammonium formate buffer of pH 3.0, 3.5, 4.0, 5.0 and ammonium acetate buffer pH 3.2 and 7.0 was studied. Chromatography was obtained by using different columns like Hypersil, C8 (100 x 4.6 mm i.d., 5µ), Hypersil, C18 (100 x 4.6 mm i.d., 5µ), Kromosil, C18 (100 x 4.6 mm i.d., 5 µ),Genesis, C18 (100 x 4.6 mm i.d., 5µ), ACE, C18 (35 x 4.6 mm i.d., 3µ), Chromolith, C18 (50 x 4.6 mm i.d., 3µ).The effect of mobile phase strength on chromatography of the analytes was evaluated by changing the organic solvent to aqueous buffer ratio. The mobile phase composition resulted in good response and peak shape was selected as the mobile phase. Effect of change in the flow rate of the mobile phase on chromatography was analyzed by changing the mobile phase flow rate of 0.4, 0.5, 0.6 and 0.8 mL/min.

4.5.6.2 Chromatographic method development and optimization

At the beginning of the method development, physicochemical properties of all analytes were studied. The reverse phase chromatography (RPC) is advantageous than the normal phase chromatography because it is more convenient, rugged, efficient, stable, simple, selective and reproducible. In bioanalysis, it is important to conduct rapid fit-for-purpose chromatographic optimization in order to achieve proper analyte retention, symmetrical peak shape and adequate response for samples containing multiple analytes.

For the optimization of the LC-MS/MS method it is necessary to identify most suitable mobile phase with proper pH and the column for achieving proper retention, peak shape, response and chromatographic resolution with other plasma interferences. Rapid mobile phase selection and then quickly applying the method to subsequent sample analyses is a challenge in drug discovery bioanalysis, where a variety of compounds from multiple studies are routinely analyzed. The reconstitution solution needs to be optimized to get good resolution and peak shape. Chromatography for BUD, FLU, PRE, PRD, DEX and TRI and IMI was conducted under different chromatographic conditions and using various columns. Mobile phase (mobile phase 1-10) ratios were varied (10, 15, 20, 25, 30, 40% of aqueous component of the mobile phase), during the mobile phase screening in order to find the most suitable

mobile phase. The ratio of aqueous-to-organic solvent is important in electrospray ionization. The efficiency of the electrospray process depends on the conductivity and surface tension of the liquid being nebulized. When the conductivity and/or the surface tension are too high (i.e. highly aqueous), it is difficult to produce a stable spray and it is difficult to vaporize the droplets formed by the action of the high voltage and nebulizing gas. The percentage of water used should not be too high since surface tension of water is much higher than the surface tension of methanol or acetonitrile. Mobile phase-10 ((ACN:0.1% formic acid in water (85:15% v/v)) showed good peak shape for FLU, PRE, PRD and DEX but very low response for the LLOQ. Mobile phase-9 ((methanol:5mM ammonium formate (92:08%v/v)) exhibited acceptable peak shape for all the analytes and good LLOQ response due to enhanced ionization of molecules at acidic pH with negligible matrix effect. The composition of the various mobile phase used in the present study were tabulated below.

Sr. No.	Organic component(A)	Aqueous component (B)
Mobile phase-1	Methanol	10 mM ammonium acetate
Mobile phase-2	Methanol	5 mM ammonium acetate
Mobile phase-3	Methanol	2 mM ammonium acetate
Mobile phase-4	Methanol	0.1% formic acid in water
Mobile phase-5	Methanol	5 mM ammonium formate
Mobile phase-6	Methanol	Water
Mobile phase-7	ACN	5 mM ammonium formate
Mobile phase-8	ACN	5 mM ammonium acetate
Mobile phase-9	Methanol	5mM Ammonium acetate pH 3.5
Mobile phase-10	ACN	0.1% formic acid

In reversed phase HPLC, the retention of analytes is related to their hydrophobicity. The more hydrophobic the analyte, longer will be the retention. When an analyte is ionized, it becomes less hydrophobic and its retention decreases. Acids lose a proton and become ionized when pH increases and bases gain a proton and become ionized when pH decreases.

To find the effect of buffer type, the analyte solution was chromatographed using ammonium acetate, ammonium formate and formic acid in water with 90% of methanol. The effect of pH

was studied using 90% v/v Methanol: 10%v/v buffer solutions having pH in the range of 2.2 to 7.0 (Ammonium acetate buffer of pH 3.5, 6.8 and 7.0 and ammonium formate buffer of pH 3.0, 4.0, 4.5 and 5.0) as the mobile phase, at a flow rate of 0.3 mL/min and ACE $C_{18,}$ (35×4.6mm,i.d., 3µ) column as the stationary phase. Peak shape of TRI and PRE were found to be asymmetrical with Genesis (100×4.6mm,i.d.,4µ) and Hypersil C18, (50×4.6mm,i.d.,5µ) column and response was also low. Peak shape was not good with the Kromacil (50×4.6mm,i.d.,4µ) column. Peak shapes with Chromolith (50×4.6mm,i.d.,3µ) column were found to be good, but the response was not consistent and the analytes were eluted faster with the flow rate of 0.4mL/min than ACE(35×4.6mm,i.d.,3µ) column. Due to early elution the interferences were observed at the retention time of the analytes.

4.5.6.3 Selection of internal standard

Chromatograms were recorded using different internal standards like Tadalafil, Telmisartan, Anastrazole and Imipramine under same chromatographic conditions to choose appropriate internal standard.

The internal standard was selected on the basis of chemical structure, polarity and solubility characteristics. The response of Imipramine was found to be reproducible with the optimized mobile phase conditions.

Based on the above trials, for the simultaneous quantification of BUD, FLU, PRE, PRD, DEX and TRI chromatographic conditions were optimized as follows:

Final chromatographic conditions

Chromatographic mode	: Reversed Phase
Isocratic/gradient mode	: Isocratic
Internal Standard	: Imipramine
Rinsing solution	: 0.1% Formic acid in methanol
Injection volume	: 25.0 µL
Column	: ACE, C18 (35×4.6mm, i.d., 3µ)
Mobile phase	: Methanol: 5mM ammonium acetate pH3.5 (90:10 %v/v)
Column oven temperature	: 40°C
Auto sampler tray temperature	: 5°C
Flush volume	: 2000 µL/Sec
Wash volume	: 2000 µL/Sec

Flow rate : 0.300 mL/min.

Run time : 3.20minutes

4.5.6.4 Selection of extraction technique

During method development different extraction techniques like liquid-liquid extraction, protein precipitation and solid phase extraction were tried.Extraction methods were optimized in order to minimize the chances of interferences in the extracted blank plasma and to remove the matrix effect. All extraction trials were done at the MQC concentration. Trials were taken to achieve consistent and precise recovery.

Protein precipitation (PPT): Different protein precipitating agents like acetonitrile, methanol, perchloric acid, hydrochloric acid and trichloroacetic acid were tried to extract the drugs efficiently form the plasma. Since protein precipitation technique dilutes the samples, it resulted in very low response. Desired LLOQ levels were not achieved for the present work. Peak shape of the analytes were not good with precipitation with ACN extraction technique due to the incompatibility of the extraction solvent with the mobile phase, when the samples were injected directly after separation. In order to overcome response and peak shape problems samples were dried (in case of methanol and acetonitrile precipitating agents) and reconstituted with the mobile phase and injected into LC-MS/MS. Response and peak shape of FLU, PRE, DEX, PRE improved but ion suppression was observed. In the next approach chromatographic conditions were changed in order to check whether elution time of matrix ions and analytes can be changed. However, this couldn't be achieved and matrix effect was observed in all trials. Different trials were undertaken by changing the volume of precipitating agents to eliminate matrix effect and interference. Matrix effect was observed due to relatively low efficiency of the technique in removing plasma proteins and co-elution of matrix phospholipids along with the analytes. In MS/MS detection systems, matrix contents have been shown to reduce the efficiency of the ionization process. The reason for ion suppression was improper sample clean up. Hence protein precipitation technique was not recommended for LC-MS/MS method.

Liquid-liquid extraction (LLE): Liquid-liquid extraction is most commonly used in bioanalytical laboratory because of its selectiveness, reproducibility and low cost when compared to solid phase extraction method. Liquid-liquid extraction (LLE) is the direct extraction of the biological material with a water-immiscible solvent. The analyte is isolated

by partitioning between the organic phase and the aqueous phase. For effective LLE, factors like solubility of the analytes in extracting solvent, low boiling point to facilitate removal of the solvent at the end of the extraction, pH of the sample (in order to allow the fractionation of the sample into acid, neutral and basic components) and viscosity of solvent to facilitate mixing with the sample matrix were considered. Rapid equilibrium was achieved by mixing using multi pulse vortexer. Selectivity was improved by choosing the least polar solvents in which the analyte was soluble.

For the present study various organic solvents were tried to obtain reproducible recovery and to remove matrix effect. In the first attempt individual organic solvents like tertiary butyl methyl ether (TBME), Diethyl ether and Ethyl acetate were tried without buffering the plasma sample. TBME showed low recovery for the analytes when used alone. The samples were basified and extracted. Recovery was improved after extracting the basified samples. But it exhibited significant matrix effect for the analytes. Ethyl acetate displayed low recovery (30 to 40%) for PRE, PRD and TRI with negligible matrix effect. Diethyl ether (DEE) showed good recovery (about 80%) but matrix effect was observed. However evaporation step was consumed more time due to low boiling point of DEE and this may leads to inconsistent recovery from batch to batch.

In the next approach mixture of organic solvents like TBME: DCM, TBME: n-hexane and TBME: ethyl acetate in different compositions like 90:10%, 80:20% and 70:30%v/v ratios were used. Buffers such as ammonium acetate, sodium bicarbonate, sodium hydroxide, formic acid were also tried. Among these samples buffered with ammonium acetate and extracted with TBME: DCM at the ratio of 70:30%v/v was found to be best solvent in terms of extraction for the analytes, but matrix effect and interferences in the blank matrix was observed for BUD, FLU and DEX at mobile phase-9. After buffering the samples with ammonium acetate and extracting the samples with TBME: DCM (70:30%v/v) about 60 to 90% recovery was obtained but matrix effect (40 to 50%) for BUD, FLU and TRI were also observed. The matrix effect was eliminated by changing the mobile phase composition, but peak shape of Triamcinolone acetonide and Imipramine were affected and also area response of BUD and FLU was reduced. Extraction conditions were not suitable for all the analytes. Therefore liquid-liquid extraction was ruled out.

Solid phase extraction (SPE): SPE was carried out to achieve higher extraction efficiency and to eliminate matrix effect. The samples were buffered with ammonium acetate, water and formic acid buffer in different trials. The effect of these buffers on recovery and matrix effect were studied. The samples from each were loaded on to previously conditioned (with 2.0mL of methanol and 2.0mL of water) Oasis HLB 1cc (30mg) cartridges. In first step of washing, 1.0mL of water was used and in the second step four different trials were taken to optimize the extraction technique with respect to recovery and matrix effect. 2.0mL 20% of methanol in water resulted in a good recovery and insignificant matrix effect. Finally analytes were eluted with 2.0mL of methanol (recovery was improved when eluted with 2.0mL when compared to 1.0mL of methanol). Eluent was evaporated to dryness under a stream of nitrogen at 40^0C. The residue was reconstituted with 80:20% v/v of methanol and 5mM ammonium acetate.25µL of the reconstitution solution was injected into LC-MS/MS system. Reconstitution with methanol resulted in peak tailing. Solid phase extraction proved to be more advantageous over protein precipitation and liquid-liquid extraction. The results of different extraction trials are presented in *figure no.10, 11, 12, and 13.*

Final extraction Procedure: To 0.475mL of blank plasma in 2.00mL micro centrifuge tube 25.0µL of analytes was spiked and vortexed to mix. 25.0 µL of internal standard working solution (1.000µg/mL) was added and vortexed to mix. 0.500mL of 10mM ammonium acetate buffer was added. This solution was loaded on 1cc HLB cartridges, previously conditioned (with 2.0mL of Methanol and 2.00mL of water). It was then washed with 2.00mL of water followed by 2.00mL of 10% v/v methanol in water and eluted with 2.0mL of methanol. The eluate was evaporated to dryness under a stream of nitrogen at 40°C. The residue was reconstituted with 80:20% v/v of methanol and 5mM ammonium acetate. 25µL of the reconstituted solution was then injected into LC-MS/MS system.

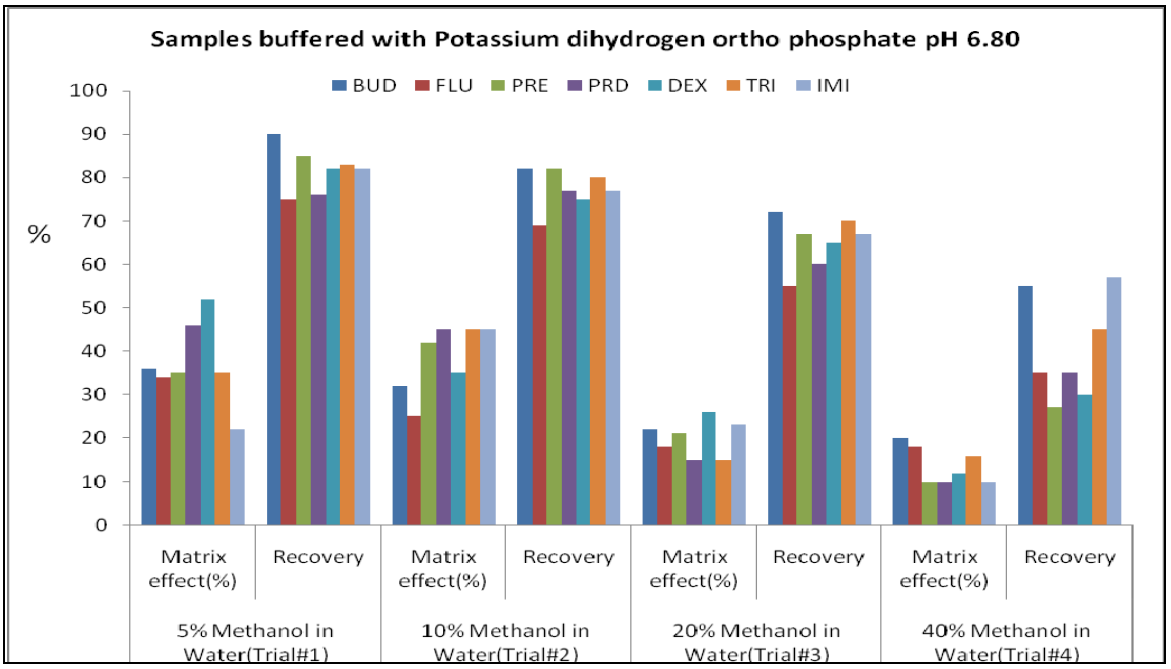

Figure No.10. Effect of potassium dihydrogen orthophosphate buffer pH 6.80 and second washing step on matrix effect and recovery of Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone, Triamcinolone acetonide and Imipramine

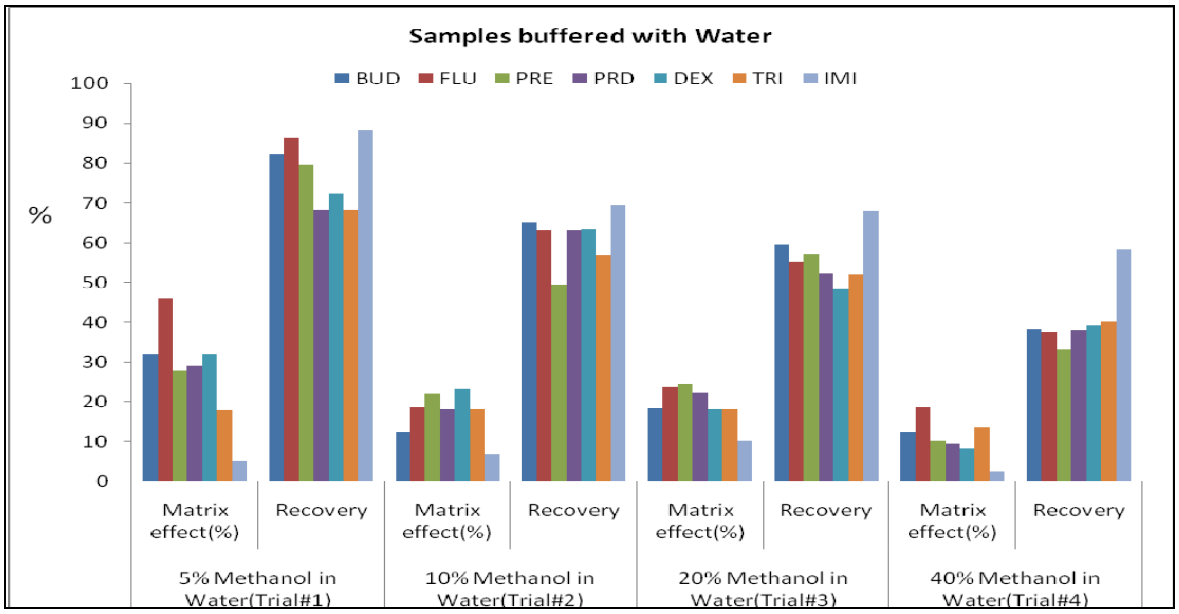

Figure No.11. Effect of water buffer and second washing step on matrix effect and recovery of Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone, Triamcinolone acetonide and Imipramine

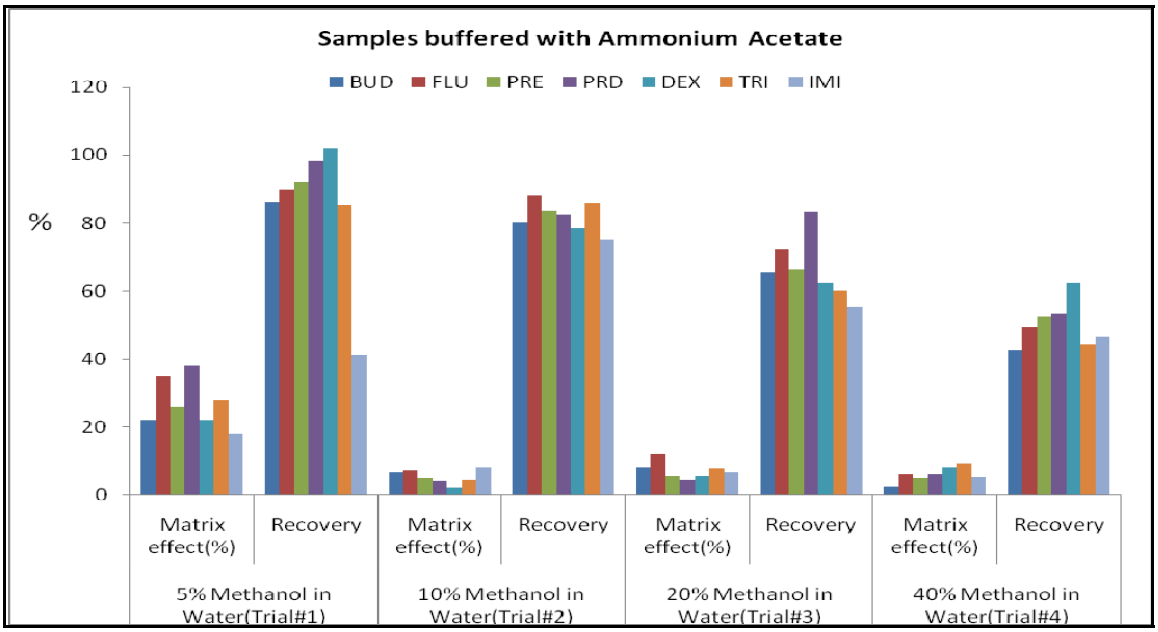

Figure No.12. Effect of ammonium acetate buffer and second washing step on matrix effect and recovery of Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone, Triamcinolone acetonide and Imipramine

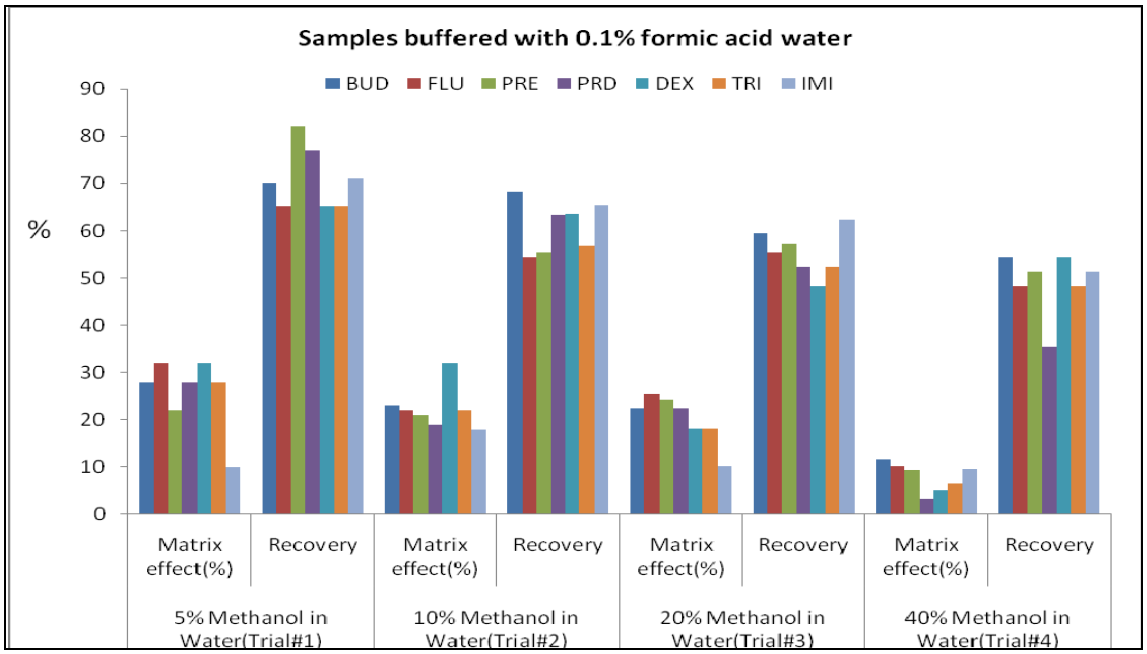

Figure No.13. Effect of 0.1% formic acid in water buffer and second washing step on matrix effect and recovery of Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone, Triamcinolone acetonide and Imipramine

4.6 Method validation procedure and results

A high performance liquid chromatographic method with mass detection for simultaneous determination of Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone and Triamcinolone acetonide was developed as per the Guidance for Industry entitled 'Bioanalytical Method Validation' of the United States Food and Drug Administration, Center for Drug Evaluation and Research (CDER) May-2001[1].

Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone and Triamcinolone acetonide and Imipramine (Internal Standard) were extracted from an aliquot of human plasma using solid phase extraction technique and injected in to a liquid chromatograph equipped with a tandem mass spectrometry detector. Quantitation was done by peak area ratio method. A weighted ($1/x^2$) linear regression was performed to determine the concentration of analytes. All regressions and figures presented in this validation report were generated by LC- Quan software version 2.5.6.

4.6.1 Chromatography

A typical chromatogram obtained from blank sample (Processed blank K_2EDTA human plasma). The representative chromatograms of lower limit of quantification and upper limit of quantification for all the analytes and internal standard are represented in *figure No.* 14(a) and (b), 15(a) and (b) and 16(a) and (b) respectively. The retention times of Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone and Triamcinolone acetonide and Imipramine are 2.10, 1.88, 1.95, 1.93, 2.45, 2.03 and 1.85 min respectively. The overall chromatographic run time is 3.20 minutes.

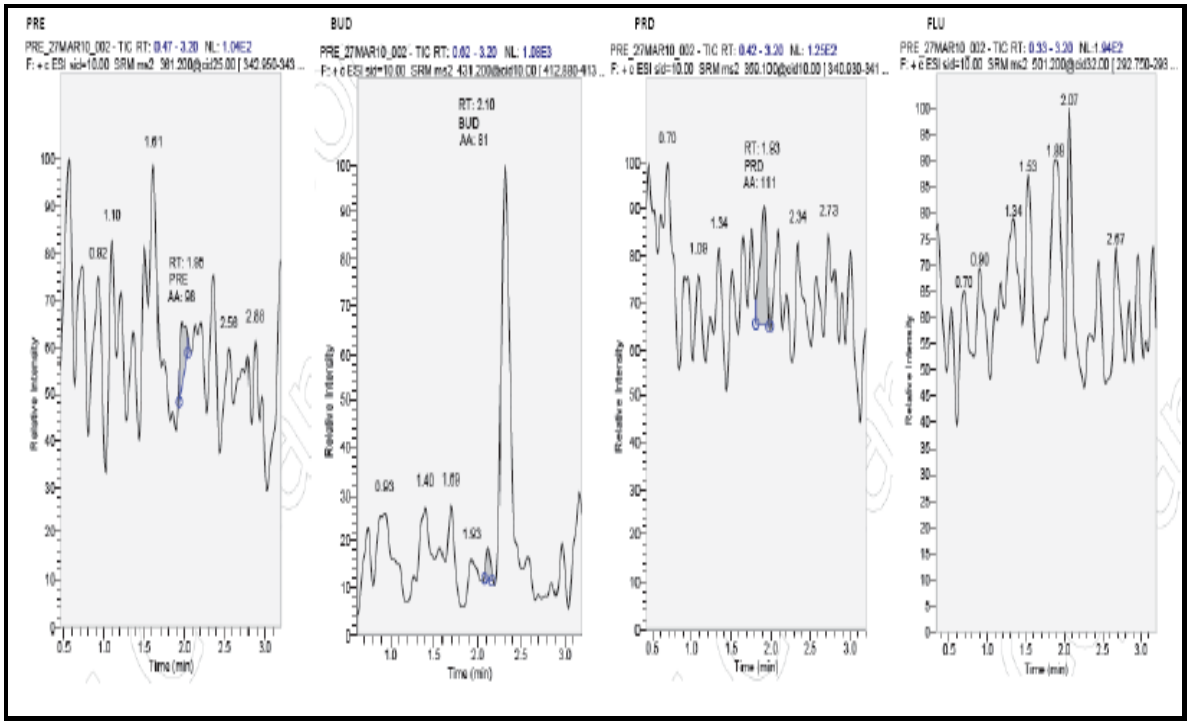

Figure No.14 (a). Representative chromatogram of Prednisolone, Budesonide, Prednisone and Fluticasone propionate in blank plasma

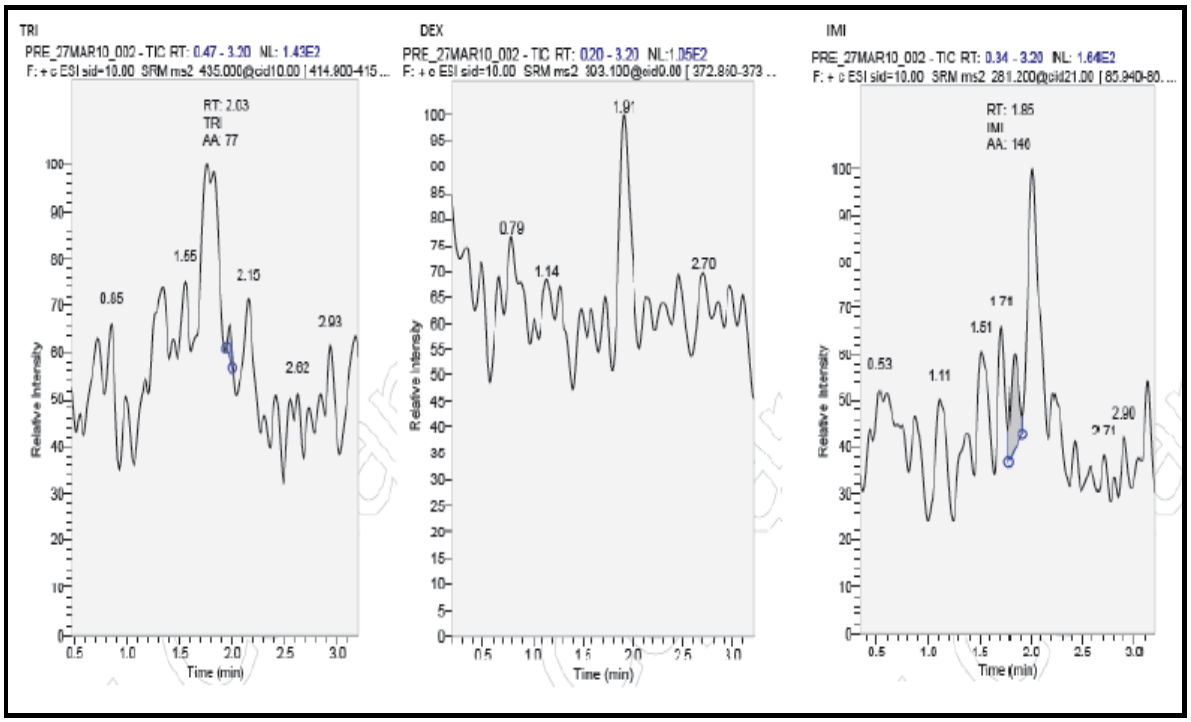

Figure. No.14 (b). Representative chromatogram of Triamcinolone acetonide, Dexamethasone and Imipramine in blank plasma

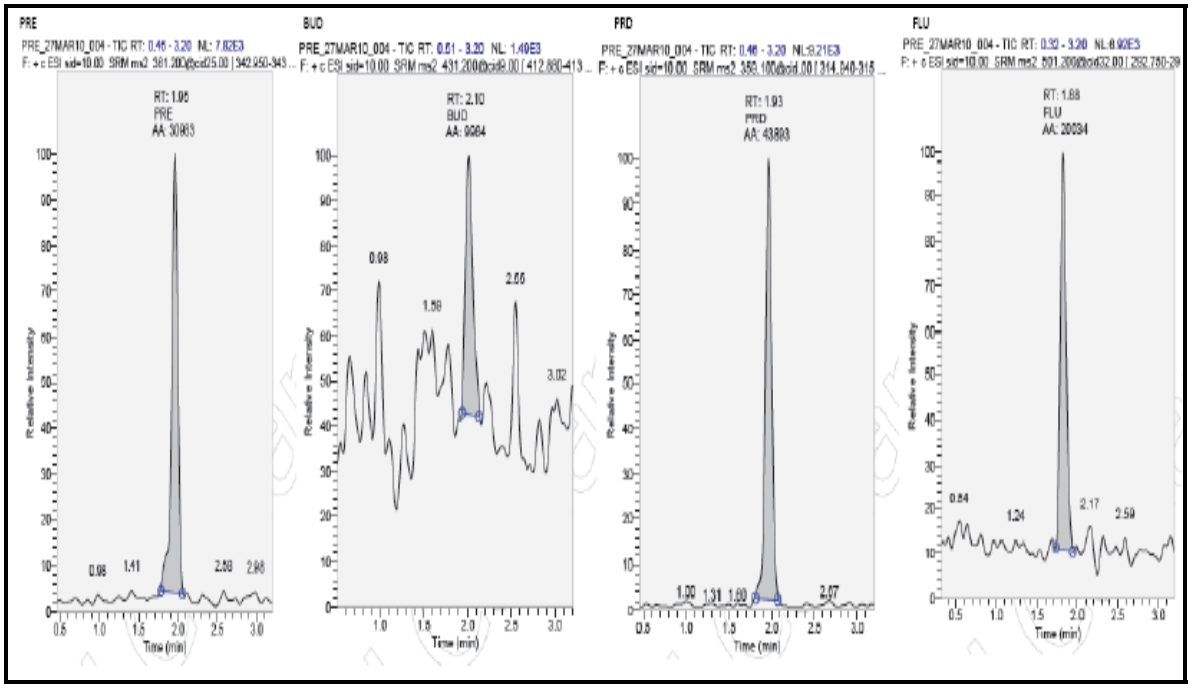

Figure No. 15(a). Representative chromatogram of Prednisolone, Budesonide, Prednisone and Fluticasone propionate in LLOQ

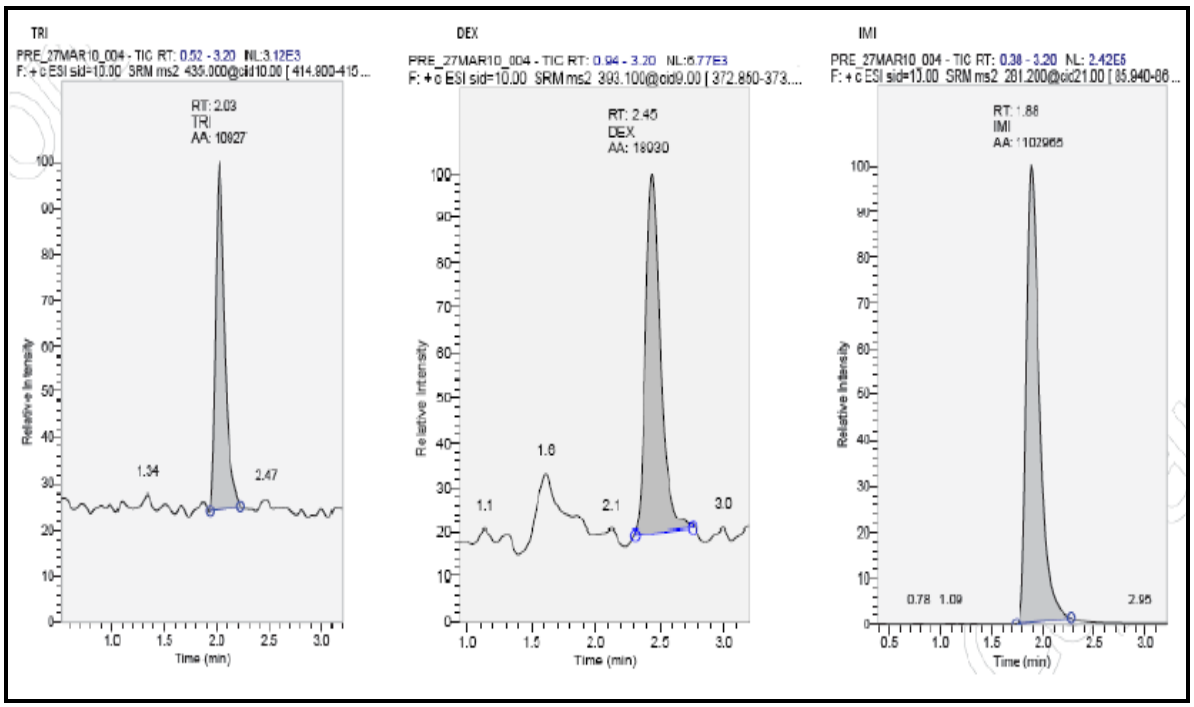

Figure No.15 (b). Representative chromatogram of Triamcinolone acetonide and Dexamethasone and Imipramine in LLOQ

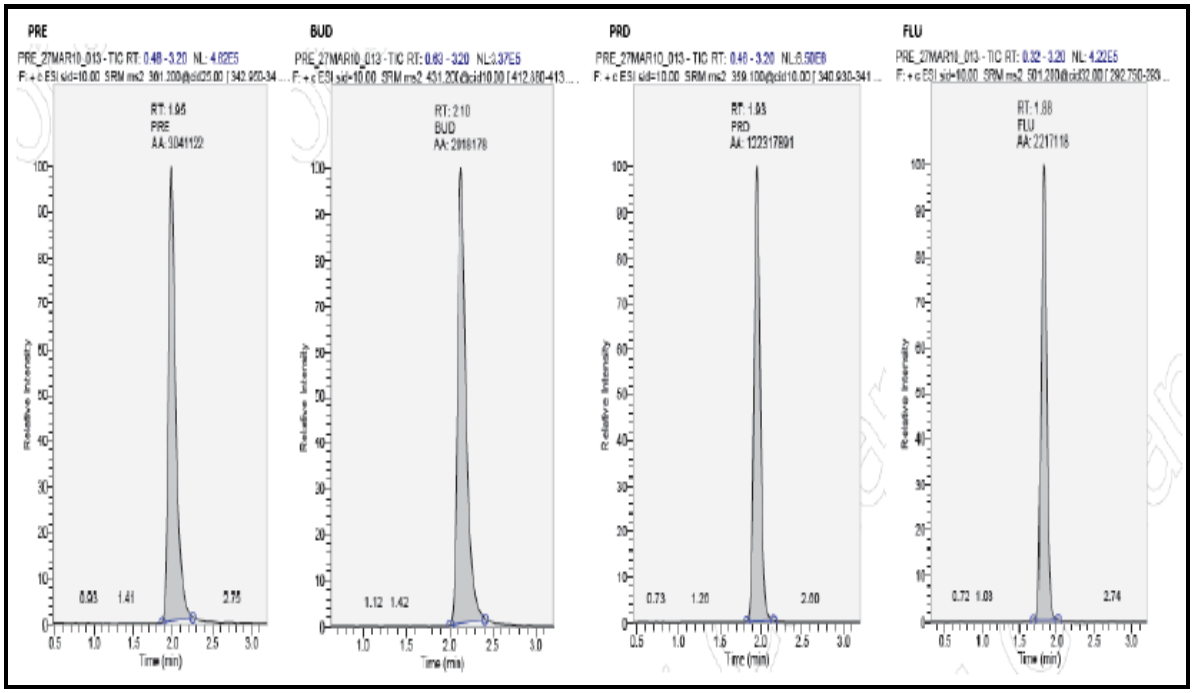

Figure No. 16(a). Representative chromatogram of Prednisolone, Budesonide, Prednisone and Fluticasone propionate in ULOQ

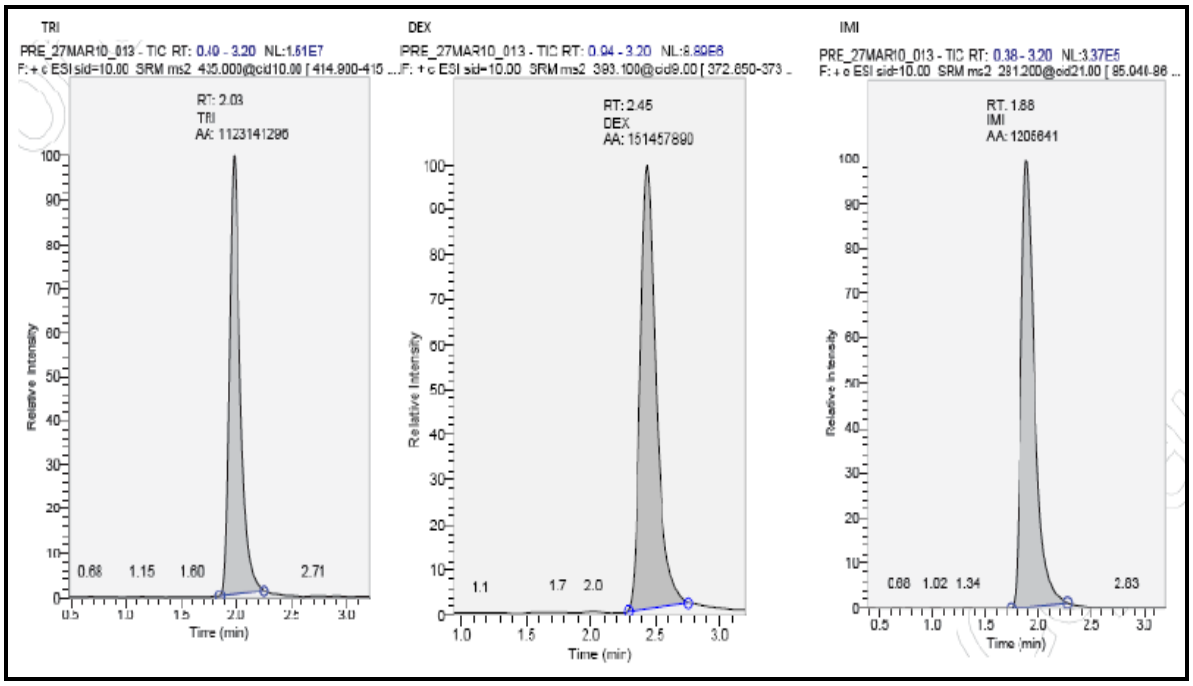

Figure No. 16(b). Representative chromatogram of Triamcinolone acetonide and Dexamethasone and Imipramine in ULOQ

4.6.2 System Suitability

System suitability was performed at the beginning of every batch. Aqueous equivalent MQC solutions of each analyte were prepared. Five injections were given and % CV for the peak area ratio was calculated.

Pre-decided acceptance criteria included %CV for peak area ratio should be ≤5.0% and %CV for retention time should be ≤2.0%.

The %CV for the peak response ratio was found to be ≤ 5% and retention time was ≤ 2. Results of system suitability are presented in the *table No. 02.*

Table No.02 System suitability results of Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone and Triamcinolone acetonide and Imipramine.

Parameters	**%CV**						
	BUD	**FLU**	**PRE**	**PRD**	**DEX**	**TRI**	**IMI**
Area Ratio (≤)	4.69	4.49	2.06	1.55	0.74	2.34	2.03
Retention Time (≤)	0.24	0.27	0.78	0.43	0.37	0.35	0.75

4.6.3 Specificity/Selectivity

Eight different lots of biological matrix (Six lots of biological matrix, one lipemic and one hemolysed with same anticoagulant) were taken. Blank plasma sample without analyte and internal standard and three extracted LLOQ samples for each lot were processed and extracted. Reconstitution solution followed by extracted blank sample and extracted LLOQ samples were analyzed.

Acceptance criteria:

a) If any peak is present at the retention time of analyte, its response should be ≤ 20% of response of the mean extracted LLOQ in each lot,

b) If any peak is present at the retention time of an internal standard, its response should be ≤ 5% of the response of a mean extracted internal standard at the concentration to be used in study and

c) A minimum of 75 % K_2EDTA lots used for the specificity should meet the above criteria.

No significant interference from the blank plasma was observed at the retention time of analytes and internal standard. Results are presented in *table no.03.*

Table No. 03. Specificity/Selectivity Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone, Triamcinolone acetonide and Imipramine

SAMPLE NAME	BUD	FLU	PRE	PRD	DEX	TRI	IMI
	Area	Area	Area	Area	Area	Area	Area
EXT-LLOQ-LOT#K2E-01-01	10783	20034	30983	43892	18930	10927	1102965
EXT-LLOQ-LOT#K2E-01-02	10098	19387	32952	40322	17892	11829	1028615
EXT-LLOQ-LOT#K2E-01-03	11092	20475	31079	41234	17345	11453	1091277
Mean	10658	19965	31671	41816	18056	11403	1074286
BLANK SAMPLE-LOT#K2E-01	982	101	1093	280	431	561	26229
% of Area	**9.21**	**0.51**	**3.45**	**0.67**	**2.39**	**4.92**	**2.44**
EXT-LLOQ-LOT#K2E-02-01	11038	21093	33092	42353	16993	12093	1083657
EXT-LLOQ-LOT#K2E-02-02	10889	20463	33017	41522	17034	13001	987625
EXT-LLOQ-LOT#K2E-02-03	10439	19272	32948	43653	18234	12034	1053761
Mean	10789	20276	33019	42509	17420	12376	1041681
BLANK SAMPLE-LOT#K2E-02	467	839	231	753	782	342	20313
% of Area	**4.33**	**4.14**	**0.70**	**1.77**	**4.49**	**2.76**	**1.95**
EXT-LLOQ-LOT#K2E-03-01	10070	22083	34973	40373	18234	11920	101354
EXT-LLOQ-LOT#K2E-03-02	11002	19857	31923	40213	17586	13002	105475
EXT-LLOQ-LOT#K2E-03-03	10569	19264	32438	42445	18268	12548	98840
Mean	10547	20401	33111	41010	18029	12490	101890
BLANK SAMPLE-LOT#K2E-03	478.00	0.00	0.00	0.00	849.00	0.00	8368
% of Area	**4.53**	**0.00**	**0.00**	**0.00**	**4.71**	**0.00**	**8.21**
EXT-LLOQ-LOT#K2E-04-01	10594	20756	33657	40734	17892	12839	97465
EXT-LLOQ-LOT#K2E-04-02	10794	21029	32664	41622	17029	11098	100149
EXT-LLOQ-LOT#K2E-04-03	11009	19843	31868	43632	18345	13110	103791
Mean	10799	20543	32730	41996	17755	12349	100468
BLANK SAMPLE-LOT#K2E-04	1000.00	873	1431	753	1211	452	8043
% of Area	**9.26**	**4.25**	**4.37**	**1.79**	**6.82**	**3.66**	**8.01**

Table No.03. Continued

SAMPLE NAME	BUD	FLU	PRE	PRD	DEX	TRI	IMI
	Area	Area	Area	Area	Area	Area	Area
EXT-LLOQ-LOT#K2E-05-01	11340	19947	34116	41233	19002	11625	1116268
EXT-LLOQ-LOT#K2E-05-02	11201	19157	32883	39645	19230	10983	996709
EXT-LLOQ-LOT#K2E-05-03	10036	19638	31534	42948	18290	12036	1083683
Mean	10859	19581	32844	41275	18841	11548	1065553
BLANK SAMPLE-LOT#K2E-05	521	421	781	732	452	983	21867
% of Area	**4.80**	**2.15**	**2.38**	**1.77**	**2.40**	**8.51**	**2.05**
EXT-LLOQ-LOT#K2E-06-01	10234	20723	32983	39567	18209	11729	1080825
EXT-LLOQ-LOT#K2E-06-02	10510	19562	30887	39476	19345	13028	1009323
EXT-LLOQ-LOT#K2E-06-03	10510	18998	31638	42893	17349	12538	1029521
Mean	10418	19761	31836	40645	18301	12432	1039890
BLANK SAMPLE-LOT#K2E-06	634	792	473	573	853	932	51254
% of Area	**6.09**	**4.01**	**1.49**	**1.41**	**4.66**	**7.50**	**4.93**
EXT-LLOQ-LOT#K2E-L-01-01	11023	21923	33927	43749	17345	11623	1049673
EXT-LLOQ-LOT#K2E-L-01-02	11234	21028	34192	42893	19034	11263	1076122
EXT-LLOQ-LOT#K2E-L-01-03	10834	20179	31573	43980	19234	11945	1104769
Mean	11030	21043	33231	43541	18538	11610	1076855
BLANK SAMPLE-LOT#K2E-L-01	786	498	743	876	347	988	20495
% of Area	**7.13**	**2.37**	**2.24**	**2.01**	**1.87**	**8.51**	**1.90**
EXT-LLOQ-LOT#K2E-H-01-01	10923	21045	30994	40998	17892	10898	976370
EXT-LLOQ-LOT#K2E-H-01-02	10345	18912	30586	41672	18302	13211	1034163
EXT-LLOQ-LOT#K2E-H-01--03	11234	22016	32110	42453	17999	12938	967520
Mean	10834	20658	31230	41708	18064	12349	992684
BLANK SAMPLE-LOT#K2E-H-01	385	748	653	849	655	874	30799
% of Area	**3.55**	**3.62**	**2.09**	**2.04**	**3.63**	**7.08**	**3.10**

4.6.4 Carry Over Check

Carryover check was performed in order to eliminate the carryover from previous injection to next injection. One aqueous equivalent ULOQ, one extracted ULOQ, one extracted blank sample, three extracted LLOQ samples were prepared from the same biological matrix lot. These samples were injected in the order of blank reconstitution solution, aqueous equivalent ULOQ, blank reconstitution solution, aqueous equivalent ULOQ, blank reconstitution solution, extracted blank sample, extracted ULOQ sample, extracted blank sample, extracted ULOQ sample, and extracted blank sample followed by three extracted LLOQ samples. Carryover was calculated by comparing the mean extracted LLOQ and extracted internal standard response to the blank reconstitution and blank sample.

Acceptance criteria:

Carry over should be ≤ 20% of mean extracted LLOQ response and ≤ 5% of extracted internal standard response.

Carryover observed in reconstitution solution and extracted blank plasma were found to be within the limit of acceptable criteria

4.6.5 Sensitivity {Lower limit of quantification (LLOQ)}

The lower limit of quantification is defined as the lowest concentration that can be determined with acceptable accuracy and precision using a particular method. This was performed by injecting six different aliquots of extracted LLOQ concentration. % deviation from the nominal concentration, % CV of the calculated concentration and Signal to noise ratio were determined.

Acceptance criteria:

a) % deviation from the nominal concentration should be within ± 20%, and

b) % CV of the calculated concentration should be ≤ 20% and

c) Signal to noise ratio should be ≥5.0

The LLOQ of BUD,FLU,PRE,PRD,DEX and TRI was 10.000 pg/mL,10.000 pg/mL, 1.000 ng/mL, 0.500 ng/mL, 0.250 ng/mL and 0.100 ng/mL respectively. Mean percentage nominal concentration was found to be 80.90%, 105.66%, 103.99%, 95.17%, 93.60% and 82.00%.

% CV was found to be 2.50%,9.33%,4.40%,2.27%,4.21% and 5.79% and S/N ratio was ≥409, ≥613, ≥424, ≥235, ≥592 and ≥170 for BUD,FLU,PRE,PRD,DEX and TRI respectively. All the results were found to be within the acceptable limits.

4.6.6 Calibration curve

The calibration curve was constructed based on the C_{max} of the individual analytes. A weighting factor $1/x^2$ was used to avoid biasing the calibration line in favour of the high standards. Calibration curve consisted of reconstitution solution, blank sample (matrix sample processed without internal standard and analyte), zero sample (matrix sample processed with internal standard) and non-zero samples (calibration standards). The back calculated concentration calibration standards, coefficient of determination (r^2) and % CV were calculated.

Acceptance criteria included each of the following

a) Area response of analyte in blank sample and zero sample should be ≤20% of STD-1,

b) Area response of ISTD in blank sample should be ≤5% of mean ISTD response,

c) The back calculated concentration of the Lower calibration standard (STD-1) must be within 80-120% of its nominal concentration,

d) The back calculated concentrations of all other calibration standards must be within 85-115% of their nominal concentration,

e) The curve must contain at least 75% of the calibration standards for evaluation of curve fitting,

f) Both STD-1 and STD-9 of the calibration curve must be within the acceptance criteria as mentioned in c) and d).

g) No two adjacent (or consecutive) calibration standards can be rejected and

h) A correlation coefficient (r^2) of the calibration curve must be ≥ 0.9800.

Calibration standards at nine concentration levels of different batches were used to choose the best calibration model for the method. The calibration model was determined by testing the algorithms linear/quadratic, 1/x weighted linear/quadratic, $1/x^2$ weighted linear/quadratic. The calibration model of $1/x^2$ weighted linear regression gave a good fit. This was the simplest, continuous and reproducible model that minimizes the bias of the back-calculated values. Results of back calculated concentrations were presented in the *table No. 4(a), (b) and (c).*

Calibration curves were found to be consistently accurate and precise for BUD, FLU, PRE, PRD, DEX and TRI over a range of 10.000 to 2000.000 pg/mL, 10.000 to 1000.000 pg/mL, 1.000 to 1000.000 ng/mL, 0.500 to 500.000 ng/mL, 0.250 to 250.000ng/mL and 0.100 to 1000.000ng/mL range respectively. The coefficient of determination is greater than or equal to 0.9956, 0.9952, 0.9943, 0.9975, 0.9976 and 0.9970 for BUD, FLU, PRE, PRD, DEX and TRI respectively. Representative calibration curves are presented the *table no.* 17(a), (b), (c), (d), (e) and (f).

Table No.4 (a).Back calculated concentrations of Budesonide and Fluticasone propionate calibration standards

Calibration Standards	Budesonide(n=4)					Fluticasone Propionate(n=4)				
	Actual Conc. (pg/mL)	Mean Conc. (pg/mL)	SD	%CV	Mean % nominal conc.	Actual Conc. (pg/mL)	Mean Conc. (pg/mL)	SD	%CV	Mean % nominal conc.
STD-1	10.000	10.343	0.682	6.59	103.43	10.000	9.96	0.069	0.69	99.60
STD-2	20.000	20.864	1.398	6.70	104.32	20.000	17.902	1.277	7.13	89.51
STD-3	100.000	102.007	6.891	6.76	102.01	50.000	44.287	2.551	5.76	88.57
STD-4	200.000	205.168	5.670	2.76	102.58	100.000	96.409	1.905	1.98	96.41
STD-5	400.000	399.236	20.849	5.22	99.81	200.000	188.969	14.24	7.54	94.48
STD-6	800.000	795.558	26.258	3.30	99.44	400.000	404.723	22.388	5.53	101.18
STD-7	1200.000	1200.573	38.132	3.18	100.05	600.000	587.123	19.541	3.33	97.85
STD-8	1600.000	1604.536	40.371	2.52	100.28	800.000	792.132	15.550	1.96	99.02
STD-9	2000.000	1971.269	50.497	2.56	98.56	1000.000	998.304	37.365	3.74	99.83

Table No.4 (b).Back calculated concentrations of Prednisolone and Prednisone calibration standards

Calibration Standards	Prednisolone(n=4)					Prednisone(n=4)				
	Actual Conc. (ng/mL)	Mean Conc. (pg/mL)	SD	%CV	Mean % nominal conc.	Actual Conc. (ng/mL)	Mean Conc. (pg/mL)	SD	%CV	Mean % nominal conc.
STD-1	10.000	9.96	0.069	0.69	99.60	0.500	0.523	0.011	2.10	104.60
STD-2	20.000	17.902	1.277	7.13	89.51	1.000	1.054	0.039	3.70	105.40
STD-3	50.000	44.287	2.551	5.76	88.57	25.000	25.941	0.229	0.88	103.76
STD-4	100.000	96.409	1.905	1.98	96.41	50.000	51.703	0.729	1.41	103.41
STD-5	200.000	188.969	14.24	7.54	94.48	100.000	104.9	4.277	4.08	104.90
STD-6	400.000	404.723	22.388	5.53	101.18	200.000	211.434	10.274	4.86	105.72
STD-7	600.000	587.123	19.541	3.33	97.85	300.000	309.696	7.128	2.30	103.23
STD-8	800.000	792.132	15.550	1.96	99.02	400.000	412.605	8.851	2.15	103.15
STD-9	1000.000	998.304	37.365	3.74	99.83	500.000	527.86	17.719	3.36	105.57

Table No.4(c).Back calculated concentrations of Dexamethasone and Triamcinolone acetonide calibration standards

Calibration Standards	Dexamethasone(n=4)					Triamcinolone acetonide(n=4)				
	Actual Conc. (ng/mL)	Mean Conc. (pg/mL)	SD	%CV	Mean % nominal conc.	Actual Conc. (ng/mL)	Mean Conc. (pg/mL)	SD	%CV	Mean % nominal conc.
STD-1	0.250	0.242	0.013	5.37	96.80	0.100	0.097	0.002	2.06	97.00
STD-2	0.500	0.500	0.018	3.60	100.00	0.200	0.196	0.009	4.59	98.00
STD-3	12.500	12.327	0.328	2.66	98.62	50.000	51.244	0.599	1.17	102.49
STD-4	25.000	25.490	0.952	3.73	101.96	100.000	96.939	7.096	7.32	96.94
STD-5	50.000	50.950	3.307	6.49	101.90	200.000	194.238	5.014	2.58	97.12
STD-6	100.000	99.384	6.470	6.51	99.38	400.000	401.971	6.512	1.62	100.49
STD-7	150.000	151.640	5.991	3.95	101.09	600.000	586.085	11.915	2.03	97.68
STD-8	200.000	199.168	19.585	9.83	99.58	800.000	807.894	10.714	1.33	100.99
STD-9	250.000	253.501	13.691	5.40	101.40	1000.000	1039.283	21.257	2.05	103.93

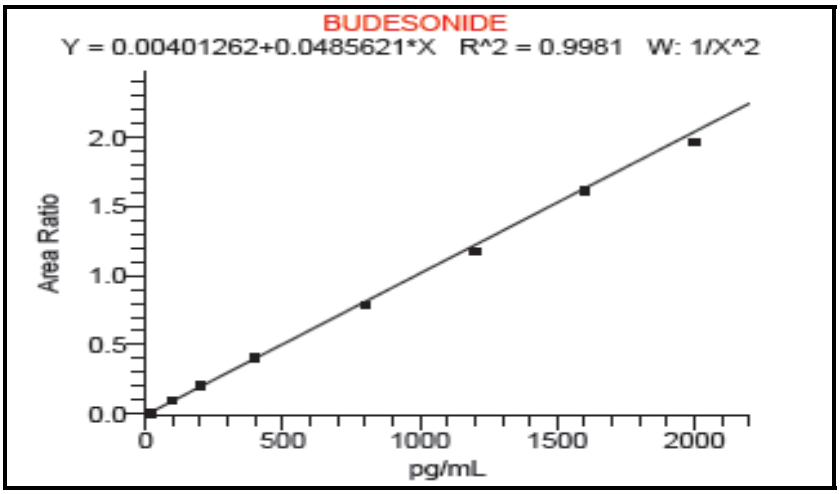

Figure No.17 (a). Representative calibration curve of Budesonide

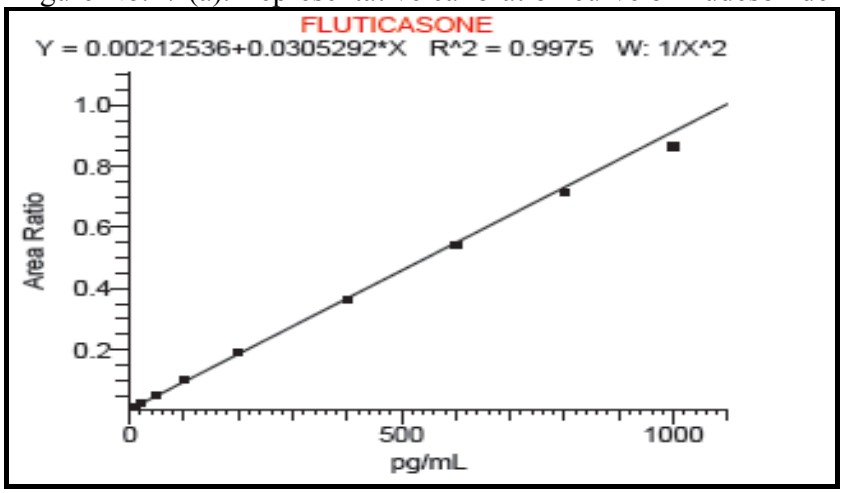

Figure No.17 (b). Representative calibration curve of Fluticasone propionate

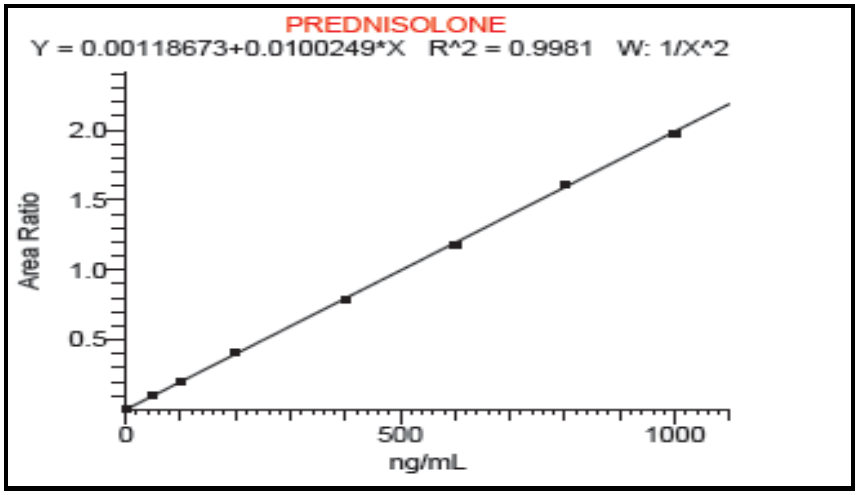

Figure No.17 (c). Representative calibration curve of Prednisolone

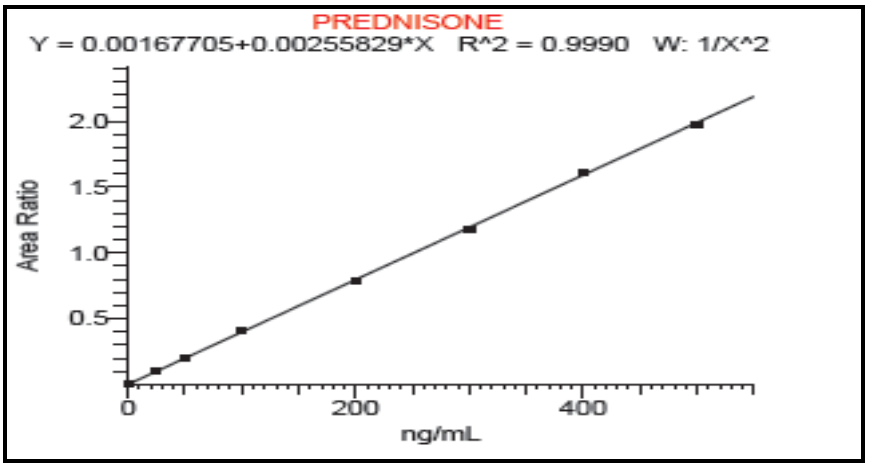

Figure No.17 (d). Representative calibration curve of Prednisone

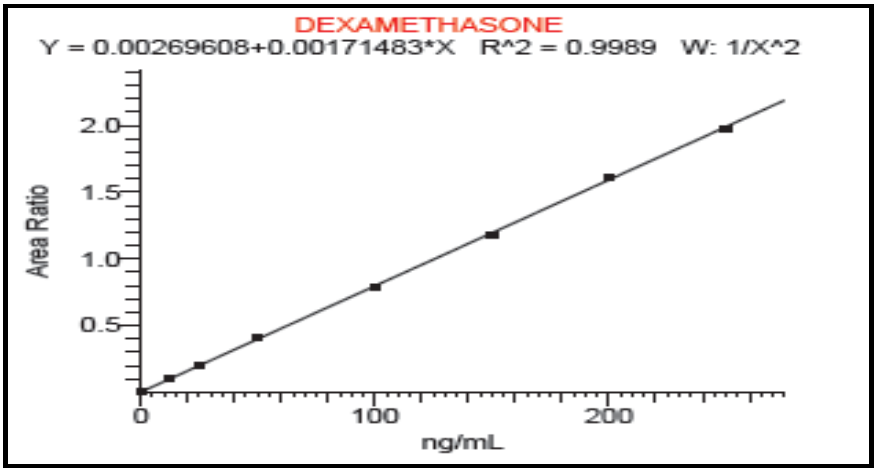

Figure No. 17 (e). Representative calibration curve of Dexamethasone

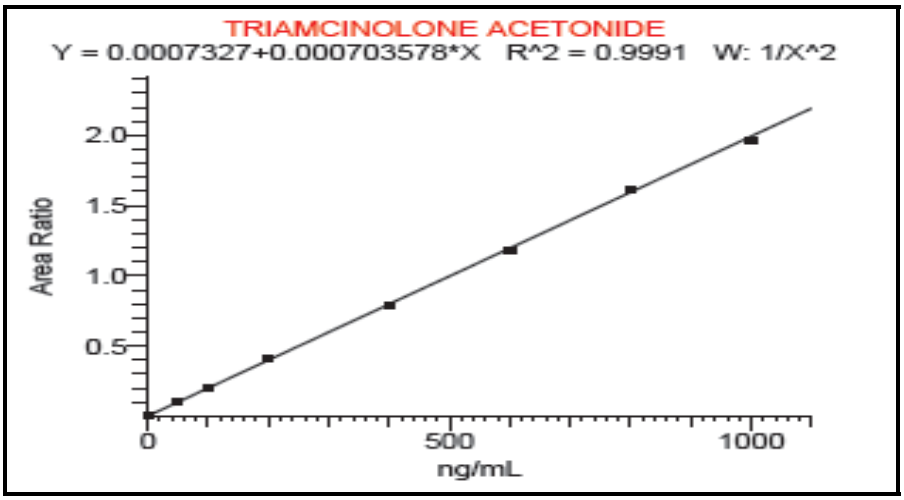

Figure No. 17(f). Representative calibration curve of Triamcinolone acetonide

4.6.7 Precision and Accuracy (P&A)

The precision of a method is defined as the closeness of agreement between independent test results obtained under prescribed conditions. Within-batch or intra-batch accuracy and precision was assessed by analyzing one calibration curve and 4 sets of QC samples (6 replicates each of the LLOQC, LQC MQC and HQC) in four different batches. Between-batch or inter-batch accuracy and precision evaluation were also assessed by analyzing 4 batches on different days. The accuracy and precision for all the batches at LLQC and LQC, MQC and HQC levels were calculated.

Acceptance criteria:

a) The global %CV for LLOQC must be ≤ 20% and for low, medium and high quality control samples must be ≤ 15% and

b) The calculated concentration of the LLOQC must be within 80-120% of its nominal concentration and for low, medium and high quality control samples must be within 85-115% of their nominal concentration

Mean percentage nominal concentration and percentage coefficient variation for all batches were found to be within the acceptance criteria in all quality control levels. Results of intra-batch accuracy and precision were presented in the *table no. 05.* Results of inter-batch accuracy and precision were presented in the *table No.06*

Table No. 05. Within-batch Precision and Accuracy of Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone and Triamcinolone acetonide

Within batch accuracy and precision					
Analyte	Batch No.	LLOQC		LQC,MQC and HQC	
		Accuracy	Precision	Accuracy	Precision
BUD	01	99.20%	3.29%	96.44 to 112.40%	≤ 7.43%
	02	100.16%	2.28%	96.75 to 101.88%	≤ 1.33%
	03	100.36%	2.53%	99.03 to 104.65%	≤ 8.47%
	04	101.44%	5.07%	90.94 to 109.34%	≤ 3.56%
FLU	01	105.66%	9.33%	92.24 to 96.08%	≤ 4.73%
	02	97.46%	6.91%	88.11 to 93.48%	≤ 5.50%
	03	86.62%	14.66%	90.00 to 98.91%	≤ 4.67%
	04	95.12%	9.34%	89.94 to 103.16%	≤ 4.92%
PRE	01	88.78%	4.94%	89.74 to 102.10%	≤ 2.15%
	02	87.63%	10.03%	94.51to 102.09%	≤ 6.60%
	03	92.22%	5.35%	91.66 to 99.14%	≤5.39%
	04	96.91%	6.28%	89.35 to 99.81%	≤ 4.39%
PRD	01	85.02%	4.50%,	93.23 to 95.17%	≤ 3.45%
	02	92.51%	9.89%,	94.90 to 99.67%	≤ 3.71%
	03	93.68%	8.17%,	94.56 to 95.86%	≤3.03%
	04	94.01%	6.19%	94.74 to 97.45%	≤ 1.98%
DEX	01	97.55%	2.51%	98.53 to 106.62%	≤ 4.31%
	02	94.12%	3.65%	98.53 to 106.37%	≤ 4.54%
	03	107.35%	7.76%	106.22 to 107.04%	≤ 6.27%
	04	91.18%	4.84%	101.96 to 109.14%	≤ 2.27%
TRI	01	94.00%	9.84%	95.20 to 101.75%	≤ 3.36%
	02	87.00%	9.77%	92.74 to 102.19%	≤5.94%
	03	84.50%	7.69%	94.86 to 98.00%	≤ 3.23%
	04	104.75%	3.58%	94.24 to102.92%	≤ 4.78%

Table No. 06. Between-batch Precision and Accuracy of Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone and Triamcinolone acetonide (n=24)

Analytes	**Actual Conc.**	**Mean Calc. Conc.**	**SD**	**%CV**	**Mean % Nominal Conc.**
Budesonide (pg/mL)	10.000	10.012	0.009	0.09	100.12
	30.000	30.408	0.879	2.89	101.36
	1000.000	1010.199	28.355	2.81	101.02
	1800.000	1851.119	32.33	1.75	102.84
Fluticasone propionate (pg/mL)	10.000	9.212	0.446	4.84	92.12
	30.000	29.254	1.757	6.01	97.51
	500.000	479.809	10.891	2.27	95.96
	900.000	894.093	18.401	2.06	99.34
Prednisolone (ng/mL)	1.000	0.971	0.022	2.27	97.05
	3.000	3.048	0.191	6.27	101.60
	500.000	510.086	16.739	3.28	102.02
	900.000	955.283	44.808	4.69	106.14
Prednisone (ng/mL)	0.500	0.485	0.011	2.27	97.05
	1.500	1.548	0.097	6.27	103.22
	250.000	255.039	12.156	4.77	102.02
	450.000	477.639	12.308	2.58	106.14
Dexamethsone (ng/mL)	0.100	0.093	0.002	2.15	92.58
	0.300	0.308	0.011	3.57	102.6
	500.000	504.676	20.242	4.01	100.94
	900.000	895.763	12.797	1.43	99.53
Triamcinolone acetonide (ng/mL)	0.100	0.100	0.001	1.00	100.13
	0.300	0.303	0.007	2.31	101.03
	500.000	511.108	14.748	2.89	102.22
	900.000	925.533	21.257	2.30	102.84

4.6.8 Recovery

Recovery of an analyte was determined for low, medium and high quality control samples. Six aliquots each of low, medium and high quality control samples and 18 aliquots of drug free biological matrix were extracted. Analytes were spiked to the reconstituted solvent of the above blank samples to obtain the post spiked LQC, MQC and HQC samples (six samples at each level (Post spiked). Post spiked and extracted quality control samples were analyzed and % recovery at each level was calculated by comparing the response area of low, medium and

high quality control levels and an internal standard. % recovery across all QC levels was also calculated.

Acceptance Criteria:

a) % CV across QC level must be ≤ 15% and

b) % CV of post spiked and extracted samples area should be ≤ 15%

Mean % recovery of Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone, Triamcinolone acetonide and Imipramine and Mean % recovery across the QC levels is presented in the *table no.07*.

Table No.07 Recovery of Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone and Triamcinolone acetonide

Analytes	QC	Mean Recovery	SD	%CV	Mean %Recovery across the QC levels
Budesonide (n=6)	LQC	79.79	3.884	4.87	77.71
	MQC	79.95	6.233	7.80	
	HQC	77.77	4.011	5.16	
Fluticasone propionate (n=6)	LQC	92.47	6.409	6.93	89.10
	MQC	87.26	8.129	9.32	
	HQC	88.44	3.324	3.76	
Prenisolone (n=6)	LQC	86.1	5.437	6.31	85.55
	MQC	83.85	4.42	5.27	
	HQC	86.7	5.427	6.26	
Prednisone (n=6)	LQC	83.55	1.952	2.34	83.24
	MQC	83.24	2.874	3.45	
	HQC	86.09	8.586	9.97	
Dexamethasone (n=6)	LQC	84.15	4.646	5.52	78.24
	MQC	73.57	65.55	3.6	
	HQC	76.99	5.165	6.71	
Triamcinolone acetonide (n=6)	LQC	85.93	9.306	10.83	88.51
	MQC	88.67	2.914	3.29	
	HQC	90.93	4.663	5.13	
Imipramine(n=18)		85.54			

4.6.9 Stability

4.6.9.1 Stock solution stability

Short term and long term solution stability for the main stock and spiking stock solutions at room temperature and at 2-8°C respectively was evaluated at MQC level. Five injections each of comparison (freshly prepared MQC equivalent concentration) and stability samples were performed. Mean % change was calculated by comparing the area of stability and comparison samples. The acceptance criteria was mean % change must be within ± 10%

4.6.9.1.1 Long term main stock and spiking stock solution stability of Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone and Triamcinolone acetonide and Imipramine for 15 days at 2-8°C.

Main Stock solution of Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone, Triamcinolone acetonide and Imipramine were prepared and aliquots of stocks were stored at 2-8°C (stability samples). MQC spiking stock solution of analytes was prepared and stored at 2-8°C (stability samples). After 15 days main stock solutions (stability samples) and spiking stock solution (stability samples) were withdrawn from 2-8°C. Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone and Triamcinolone acetonide aqueous equivalent MQC spiking stock and working solution of Imipramine were prepared from the main stock (stability samples) and these stability samples were analyzed with the freshly prepared aqueous equivalent MQC spiking stock and working solution of internal standard. Area response of stability samples and freshly prepared samples were compared to determine mean % change during stability period. Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone and Triamcinolone acetonide and Imipramine main stock solution and spiking stock solution was found to be stable at 2-8°C for 15 days. The results were presented in the *table no. 08 and 09.*

Table No. 08 Main stock solution stability of Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone, Triamcinolone acetonide and Imipramine for 15 days at 2-8ºC

Analytes	Mean area of comparison sample	Mean area of Stability sample	Mean % Change
BUD	1398822	1358656	-2.87
FLU	1135241	1151447	1.43
PRE	1912385	1958864	2.43
PRD	81235967	80482687	-0.93
DEX	115455623	114190874	-1.10
TRI	68562356	71523214	4.32
IMI	2012365	1963326	-2.44

Table No. 09. Spiking stock solution stability of Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone and Triamcinolone acetonide for 15 days at 2-8ºC

Analyte	Mean area of comparison sample	Mean area of Stability sample	Mean % Change
BUD	1398822	1353195	-3.26
FLU	1135241	1089568	-4.02
PRE	1912385	1816571	-5.01
PRD	81235967	79481487	-2.16
DEX	115455623	114809824	-0.56
TRI	68562356	67481148	-1.58

4.6.9.1.2 Short term main stock and spiking stock solution stability of Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone and Triamcinolone acetonide and Imipramine for 13 hrs at room temperature

Main stock solution of Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone, Triamcinolone acetonide and Imipramine were prepared and kept at room temperature for 13 hrs (stability sample). MQC spiking stock solution of analytes and internal standard working solution were prepared and kept at room temperature for 13 hrs (stability sample). After 13 hrs aqueous equivalent MQC spiking stock solution of Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone and Triamcinolone acetonide were prepared from the main stock (stability sample) and MQC spiking stock solution and working solution of internal standard(stability samples) were analyzed against freshly prepared samples (comparison samples). Area of stability samples and freshly prepared samples were compared to determine mean % change during stability period. Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone and

Triamcinolone acetonide main stock and spiking stock solution working solution and Imipramine main stock and working solutions were found to be stable at room temperature for 13 hrs. The results were presented in the *table no.10 and 11.*

Table No.10. Main stock solution stability of Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone, Triamcinolone acetonide and Imipramine 13 hrs at room temperature

Analyte	Mean area of comparison sample	Mean area of Stability sample	Mean % Change
BUD	1398822	1400456	0.12
FLU	1135241	1135702	0.04
PRE	1912385	1947167	1.82
PRD	81235967	80813247	-0.52
DEX	115455623	116103584	0.56
TRI	68562356	66783556	-2.59
IMI	2012365	2021862	0.47

Table No.11. Spiking stock solution stability of Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone, Triamcinolone acetonide and Imipramine 13 hrs at room temperature

Analyte	Mean area of comparison sample	mean area of Stability sample	Mean % Change
BUD	1398822	1333764	-4.65
FLU	1135241	1115956	-1.70
PRE	1912385	1837841	-3.90
PRD	81235967	78229482	-3.70
DEX	115455623	114443574	-0.88
TRI	68562356	66968845	-2.32
IMI	2012365	1989986	-1.11

4.6.9.2 Stability of drug in plasma

The stability of the analytes in the plasma was analyzed at different conditions anticipated during the clinical/pharmacokinetic studies like bench top stability, wet extract stability, dry extract stability, process stability, freeze thaw stability, In-injector stability and long term stability.

Acceptance criteria for the plasma stability samples

The acceptance criteria would be the same for bench top stability, post preparative stability, freeze thaw stability, dry extract stability, wet extract stability, process stability, which are:-

a) The calculated concentration of the LQC and HQC must be within 85-115% of its nominal concentration and

b) Mean % change must be within ± 15%

4.6.9.2.1 Bench top stability: Bench top (BT) stability was evaluated to confirm that analyte degradation does not occur during time of exposing to the room temperature, which is based on the expected duration the samples will be maintained at room temperature during the study. Bench Top stability was determined at low and high quality control levels. Samples were prepared at low and high quality control levels and kept at bench top at room temperature for a minimum of four hours (stability samples). After this time, fresh calibration standards and quality control samples (comparison samples) were prepared, extracted and analyzed with the stability samples. Mean % change was calculated.

Six samples of LQC and HQC were spiked in K_2EDTA plasma and were kept at room temperature for 12 hrs and were processed and analyzed along with freshly prepared calibration standards, LQC and HQC samples. Concentrations were compared to determine mean % change during stability period. From the results the analytes were found to be stable for 12 hrs at room temperature. The results were presented in the *Table No. 12.*

Table No. 12. Bench top stability of Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone and Triamcinolone acetonide for 12 hrs at room temperature

Analyte	**Level**	**Fresh Sample Conc.(ng/mL)**	**Stability Sample Conc. (ng/mL)**	**Mean % Change**
BUD	LQC(n=6)	33.234	31.895	-4.03
	HQC(n=6)	1903.137	1833.381	-3.67
FLU	LQC(n=6)	29.652	29.287	-1.23
	HQC(n=6)	886.321	849.601	-4.14
PRE	LQC(n=6)	3.451	3.367	-2.44
	HQC(n=6)	935.399	965.601	3.23
PRD	LQC(n=6)	1.502	1.424	-5.21
	HQC(n=6)	458.033	447.298	-2.34
DEX	LQC(n=6)	0.324	0.338	4.43
	HQC(n=6)	924.132	944.010	2.15
TRI	LQC(n=6)	0.325	0.339	4.43
	HQC(n=6)	945.235	965.528	2.15

4.6.9.2.2 Freeze and Thaw stability (after 4th cycle at -70 ±5°C)

From a practical standpoint, it is often necessary to subject samples to multiple freeze-thaw cycles before reportable analytical results may be obtained. Freeze thaw (FT) stability has been performed at LQC and HQC Levels. Samples were exposed to four freeze thaw cycles before subjecting them to freeze and thaw stability. Stability samples were analyzed with fresh calibration standards along with low and high quality control samples (comparison samples). Comparison samples and stability samples were analyzed with the calibration standards and mean % change was calculated. Acceptance criteria were the same as bench stop stability.

Four sets of LQC and HQC level samples were aliquoted and frozen at -70±5°C. Six samples from each concentration were subjected to four freeze and thaw cycles (stability samples). These samples were processed and analyzed along with freshly prepared calibration standards, LQC and HQC samples (comparison samples). Concentrations were compared to determine mean % change after 4th cycle. The results have shown Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone and Triamcinolone acetonide were found to be stable after four cycles of freeze and thaw cycles at -70±5°C. The results were presented in the *table No. 13.*

Table No. 13. Freeze thaw stability of Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone and Triamcinolone acetonide after 4 cycles

Analyte	**Level**	**Fresh Sample Conc.(ng/mL)**	**Stability Sample Conc. (ng/mL)**	**Mean % Change**
BUD	LQC(n=6)	31.997	29.829	-6.78
	HQC(n=6)	1839.771	1772.177	-3.67
FLU	LQC(n=6)	28.135	26.891	-4.42
	HQC(n=6)	902.032	837.231	-7.18
PRE	LQC(n=6)	2.833	2.795	-1.34
	HQC(n=6)	895.567	834.831	-6.78
PRD	LQC(n=6)	1.499	1.415	-5.60
	HQC(n=6)	467.805	440.990	-5.73
DEX	LQC(n=6)	0.306	0.320	4.64
	HQC(n=6)	914.235	904.178	-1.10
TRI	LQC(n=6)	0.331	0.346	4.64
	HQC(n=6)	885.226	875.495	-1.10

4.6.9.2.3 Dry-extract (DE) stability: Dry-extract stability was carried out whenever the sample processing involves evaporation before injecting in to chromatographic system to anticipate the sample exposure to room temperature after evaporation. Dry-extract stability is determined at low and high quality control levels. Samples were processed and after the drying step, samples were kept at room temperature for a minimum of 4 hours (stability samples). Stability samples were analyzed with fresh calibration standards along with low and high quality control samples (comparison samples). Comparison samples and stability samples were analyzed with the calibration standards and mean % change was calculated. Acceptance criteria were the same as bench stop stability.

Six samples of LQC and HQC were spiked in K_2EDTA plasma and processed. After drying, samples were kept at room temperature for 4 hrs and were reconstituted and analyzed along with freshly prepared calibration standards, LQC and HQC samples. Concentrations were compared to determine mean % change during stability period. The dry extract of the analytes were found to be stable for 4 hrs at room temperature. The results were presented in the *table no. 14.*

Table No. 14. Dry extract stability of Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone and Triamcinolone acetonide for 4 hrs at room temperature

Analyte	**Level**	**Fresh Sample Conc.(ng/mL)**	**Stability Sample Conc. (ng/mL)**	**Mean % Change**
BUD	LQC(n=6)	27.466	27.796	1.2
	HQC(n=6)	1733.108	1699.707	-1.93
FLU	LQC(n=6)	29.302	27.836	-5.00
	HQC(n=6)	896.302	875.110	-2.36
PRE	LQC(n=6)	3.215	3.100	-3.56
	HQC(n=6)	886.032	867.801	-2.06
PRD	LQC(n=6)	1.496	1.445	-3.56
	HQC(n=6)	465.235	458.928	-1.36
DEX	LQC(n=6)	0.278	0.266	-4.02
	HQC(n=6)	850.235	822.635	-3.25
TRI	LQC(n=6)	0.295	0.283	-4.02
	HQC(n=6)	910.325	880.735	-3.25

4.6.9.2.4 Wet-extract (WE) stability: Wet-extract stability is determined at low and high quality control levels. LQC and HQC samples were prepared, processed and kept at room temperature (stability samples) after reconstitution/elution. Stability samples were analyzed

with fresh calibration standards along with low and high quality control samples (comparison samples). Comparison samples and stability samples were analyzed with the calibration standards and mean % change was calculated. The acceptance criteria were the same as bench stop stability.

Six samples of LQC and HQC were spiked in K_2EDTA plasma and processed. After reconstitution, samples were kept at room temperature for 6 hrs and were analyzed along with freshly prepared calibration standards, LQC and HQC samples. Concentrations were compared to determine mean % change during stability period. The analytes were found to be stable after reconstitution for 6 hrs kept at room temperature. The results were presented in the *table no. 15*

Table No. 15. Wet extract stability of Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone and Triamcinolone acetonide for 6 hrs at room temperature

Analyte	**Level**	**Fresh Sample Conc.(ng/mL)**	**Stability Sample Conc. (ng/mL)**	**Mean % Change**
BUD	LQC(n=6)	30.329	29.059	-4.19
	HQC(n=6)	1910.820	1799.002	-5.85
FLU	LQC(n=6)	33.201	32.791	-1.23
	HQC(n=6)	916.235	893.194	-2.51
PRE	LQC(n=6)	2.885	3.193	10.67
	HQC(n=6)	946.056	895.715	-5.32
PRD	LQC(n=6)	1.403	1.353	-3.54
	HQC(n=6)	440.023	422.019	-4.09
DEX	LQC(n=6)	0.295	0.280	-5.06
	HQC(n=6)	902.617	872.902	-3.29
TRI	LQC(n=6)	0.286	0.273	-5.06
	HQC(n=6)	863.265	834.902	-3.29

4.6.9.2.5 Process stability: Process stability is determined at low and high quality control levels to prove the stability of the analyte/s during sample processing i.e., after addition of buffer, reagent and extraction solvent before evaporation/elution. One set of LQC and HQC were kept at room temperature for 4 hrs at each level of sample processing steps like after adding buffer, after vortexing, after centrifugation, separated supernatant, before evaporation (stability samples). After 4 hours, fresh calibration standards and one set of low and high quality control samples were prepared and extracted (comparison samples) along with stability samples. Comparison and stability samples along with freshly prepared calibration

standards were analyzed. Mean % change in stability samples was determined. The acceptance criteria were the same as bench stop stability.

Six samples of LQC and HQC were spiked in K_2EDTA plasma and kept at room temperature after adding buffer for 2 hrs. Before drying the samples were kept at room temperature for 2 hrs and then subjected to drying and reconstitution. These samples were analyzed along with freshly prepared calibration standards, LQC and HQC samples. Concentrations were compared to determine mean % change during stability period. The samples were found to be stable for 4 hrs during the samples processing steps.The results were presented in the *table no. 16.*

Table No.16. Process stability of Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone and Triamcinolone acetonide for 4 hrs at room temperature

Analyte	**Level**	**Fresh Sample Conc.(ng/mL)**	**Stability Sample Conc. (ng/mL)**	**Mean % Change**
BUD	LQC(n=6)	30.361	28.908	-4.79
	HQC(n=6)	1829.998	1801.184	-1.57
FLU	LQC(n=6)	28.024	26.335	-6.03
	HQC(n=6)	895.032	859.032	-4.02
PRE	LQC(n=6)	2.985	2.665	2.57
	HQC(n=6)	856.481	842.999	-5.67
PRD	LQC(n=6)	1.564	1.498	-4.23
	HQC(n=6)	430.025	421.345	-2.02
DEX	LQC(n=6)	0.330	0.326	-1.23
	HQC(n=6)	886.023	841.499	-5.03
TRI	LQC(n=6)	0.321	0.317	-1.23
	HQC(n=6)	852.362	809.499	-5.03

4.6.9.2.6 In-injector stability at 5°C in auto sampler for 49 hrs

Stability of processed samples in the instrument over the anticipated run time needs to be assessed as in case of instrument failure. In-injector stability was determined at low and high quality control levels. Stability samples were analyzed with fresh calibration standards along with low and high quality control samples (comparison samples). Comparison samples and stability samples were analyzed with the calibration standards and mean % change was calculated.

Six samples each of LQC and HQC samples were prepared and processed. These processed samples were kept in auto sampler for 49 hrs at 5°C. After 49 hrs the samples were analyzed

along with freshly prepared calibration standards, LQC and HQC samples. Concentrations were compared to determine mean % change during stability period. Results have shown that the analytes were stable for 49 hrs at 5°C (in auto sampler). The results were presented in the *table no. 17.*

Table No. 17. In-injector stability of Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone and Triamcinolone acetonide for 49 hrs at 5°C

Analyte	**Level**	**Fresh Sample Conc.(ng/mL)**	**Stability Sample Conc. (ng/mL)**	**Mean % Change**
BUD	LQC(n=6)	28.223	29.542	4.67
	HQC(n=6)	1748.046	1794.726	2.67
FLU	LQC(n=6)	31.056	31.940	2.85
	HQC(n=6)	910.024	958.572	5.33
PRE	LQC(n=6)	3.011	3.054	1.44
	HQC(n=6)	925.032	940.182	1.64
PRD	LQC(n=6)	1.409	1.429	1.44
	HQC(n=6)	489.235	483.199	-1.23
DEX	LQC(n=6)	0.321	0.306	-4.56
	HQC(n=6)	926.032	851.808	-8.02
TRI	LQC(n=6)	0.289	0.276	-4.56
	HQC(n=6)	950.235	873.992	-8.02

4.6.9.2.7 Long term stability: Long-term storage stability assessment experiments are designed to confirm analyte stability in the test system matrix covering the length of time from sample collection to sample analysis. Such an assessment gives credibility to the final study data. Long term stability was determined at LQC and HQC levels. Stability samples were stored at -70±5°C for 90 days. Long term stability was assessed by analyzing extracted fresh calibration standards and one set of low and high quality control samples (comparison samples) with stability samples. Mean % change of stability samples was determined. The analytes were found to be stable 90 days at -70 ±5°C. Results were presented in *table no. 18*.

Table No. 18. Long-term stability of Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone and Triamcinolone acetonide for 90 Days at -70 ±5°C.

Analyte	**Level**	**Fresh Sample Conc.(ng/mL)**	**Stability Sample Conc. (ng/mL)**	**Mean % Change**
BUD	LQC(n=6)	27.896	25.446	-8.78
	HQC(n=6)	1792.697	1669.111	-6.89
FLU	LQC(n=6)	32.021	29.246	-8.67
	HQC(n=6)	886.032	839.012	-5.32
PRE	LQC(n=6)	3.224	3.291	2.07
	HQC(n=6)	873.512	836.341	-4.26
PRD	LQC(n=6)	1.582	1.574	-0.52
	HQC(n=6)	420.555	405.003	-3.70
DEX	LQC(n=6)	0.324	0.317	-2.44
	HQC(n=6)	911.500	909.123	-0.26
TRI	LQC(n=6)	0.265	0.258	-2.44
	HQC(n=6)	854.250	852.028	-0.26

4.6.10 Dilution integrity

In order to anticipate the sample concentrations which exceed the upper limit of quantitation or anticipated insufficient sample volume in the study samples a test for sample dilution with blank matrix should be performed. This was carried out by spiking 2xULOQ spiking stock solution in biological matrix to $^1/_2$ and $^1/_4$ dilute samples. Six aliquots each of diluted samples and calibration standards were processed, extracted and analyzed. %CV and accuracy was calculated.

Acceptance criteria:

a) % CV of diluted samples must be ≤ 15% and

b) % deviation from the nominal concentration should be between 85 to 115%.

The mean percentages nominal concentration was found to be within ± 10% for both ½ dilution and ¼ dilution to all analytes . The %CV was found to be ≤4.64, ≤2.83, ≤3.20, ≤2.46,≤9.65 and ≤2.14 for . BUD, FLU, PRE, PRD, DEX and TRI. This indicated the integrity of the analyte results after the ½ dilution and ¼ dilution.

4.6.11 Matrix effect

The presence of unmonitored, co-eluting compounds from the matrix may affect the detection of analytes. Matrix effect was measured in six different lots of same biological matrix at LQC

and HQC levels in triplicate. Matrix Factor was calculated by comparing the peak response in presence of matrix ions to that of peak response in absence of matrix ions. Internal standard-normalized Matrix Factor for the analyte was also calculated.

a) % CV across different lots should be less than 15%

b) Matrix effect should be within ±20%

No significant matrix effect was observed at LQC and HQC levels of Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone and Triamcinolone acetonide and Imipramine. The results were found to be within ±20% for all analytes

4.6.12 Hemolysis and Lipemic Effect

Hemolysis and Lipemic effect is performed to check the effect of hemolysed and lipemic plasma on analytical results. Six aliquots each LQC and HQC samples were prepared in hemolysed plasma and lipemic plasma with same anticoagulant (K_2EDTA). Calibration standards in biological matrix was prepared, processed and extracted with the QC samples. The % CV and mean % deviation from the nominal concentration of LQC and HQC samples was reported.

Acceptance criteria

a) The %CV for low and high quality control samples must be ≤ 15%

b) The calculated concentration for low and high quality control samples must be within 85-115% of their nominal concentration

Hemolised and lipemic effect results are found to be within the acceptance criteria. Therefore hemolised and lipemic samples will not have any impact on the quantification of the analytes. The results are given in the *table No.19*

Table No.19. Hemolytic and lipemic effect of Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone and Triamcinolone acetonide.

	Level	**BUD**	**FLU**	**PRE**	**PRD**	**DEX**	**TRI**
Hemolytic effect	LQC (Mean % nominal conc.)	103.95	104.14	92.59	100.61	99.35	95.58
	HQC (Mean % nominal conc.)	101.64	103.19	101.12	104.85	104.38	103.17
	%CV	≤7.07	≤3.50	≤6.18	≤7.08	≤4.81	≤6.72
Lipemic effect	LQC (Mean % nominal conc.)	89.33	106.31	88.41	96.78	106.06	95.91
	HQC (Mean % nominal conc.)	92.7	104.74	99.86	97.90	103.11	99.23
	%CV	≤7.75	≤7.34	≤4.40	≤4.60	≤6.17	≤5.35

4.6.13 Ruggedness

Ruggedness of an analytical procedure is a measure of its capacity to remain unaffected by deliberate variations in method parameters and provides an indication of its reliability during method usage. Ruggedness was carried out by analyzing two P&A batches prepared by different analysts, one P&A batch was analyzed with different column of the same type (different lot) and one P&A batch was analyzed with reagents of different manufacturer or lot number. %CV and mean % nominal concentration were calculated in each case.

Acceptance criteria:

a) The %CV for LLOQC must be ≤ 20% and for low, medium and high quality control samples, it must be ≤ 15%,

b) The calculated concentration of the LLOQC must be within 80-120% of its nominal concentration and for low, medium and high quality control samples it must be within 85-115% of their nominal concentration and

c) The global %CV for LLOQC must be ≤ 20% and for low, medium and high quality control samples, it must be ≤ 15%

The results from all four P&A batches were found to be within the acceptance criteria. the mean percentage nominal concentration was found to be within ± 11.26% and the % CV was found to be ≤ 9.64% at all QC levels. The method was proven to be rugged, even with the external variations like analysts, make of reagents and change in lot numbers of the columns.

4.6.14 Production batch run (Precision and Accuracy).

Production batch run precision and accuracy is performed to check the precision and accuracy of expected length of run during study sample analysis. Minimum 5% of QC samples (LQC, MQC and HQC) to that of total number of samples were processed and analyzed along with the calibration standards. %CV and mean % nominal concentration were calculated.

Acceptance criteria

a) The %CV for low, medium and high quality control samples must be ≤ 15%,
b) The calculated concentration for low, medium and high quality control samples must be within 85-115% of their nominal concentration,
c) At least 50% QCs must fall within above mentioned criteria at each level and
d) Overall 67% of QCs must fall within above mentioned criteria

The production batch compromising of 160 samples was analyzed with production run QC samples. Mean % nominal concentration was found to be 91.07 to 106.54 and the %CV was ≤9.18. The results have shown to be about 160 samples will be analyzed in a single batch which results in more number of sample analysis per day.

4.6.15 Reinjection Reproducibility

Reinjection reproducibility was evaluated to determine if an analytical run could be reanalyzed in the case of instrument failure. Six aliquots each of LLOQC, LQC, MQC and HQC samples and calibration standards were processed, extracted and analyzed. Samples were re-injected after a minimum of 12hrs. % difference of the mean calculated concentration between two batches at all QC levels was determined. Results are presented in *Table No. 38*.

Acceptance criteria:

a) The %CV for LLOQC must be ≤ 20% and for low, medium and high quality control samples must be ≤ 15%,

b) The calculated concentration of the LLOQC must be within 80-120% of its nominal concentration and for low, medium and high quality control samples must be within85-115 % of their nominal concentration and

c). The mean percentage change of the calculated concentration should be within ±20% for LLOQC and ±15% for low, medium and high quality control samples.

One P&A batch was re-injected after 12 hrs of the initial analysis. The mean % change between the initial calculated concentration and the calculated concentration of the re-injected samples at LLOQC, LQC, MQC and HQC was found to be within 5%. This indicated the samples re-injection will not have any impact on the final outcome of the results.

4.7 Conclusion

Corticosteroids are most frequently prescribed drugs for anti-inflammatory and immune-modulatory effects. Systemic use of corticosteroids is associated with significant side effects and low dose corticosteroids may provide a favorable benefit/risk ratio than higher doses for the therapeutic applications. This calls for quantification of the drug at very low concentrations in body fluids. Various methods have been reported for the estimation of synthetic corticosteroids in plasma, serum and urine. However due to the pleiotropic pharmacological effects of the corticosteroids and the high potency of some of these agents,

systemic levels must be determined to ensure safety of the therapy. Developing a method for the simultaneous estimation of several synthetic corticosteroids is a challenging task, owing to different ionization efficiencies of the analytes, matrix effect and recovery. A method has been developed and validated for six clinically important synthetic corticosteroids Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone and Triamcinolone acetonide.

A simple, rapid and rugged HPLC–MS/MS method was developed and validated for the determination of Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone and Triamcinolone acetonide in human K_2EDTA plasma. The method was validated in accordance with USFDA guidelines "Guidance for the Industry: Bioanalytical Method Validation, 2001". The mass spectra for each analyte were obtained individually, and the most abundant product ions were selected for SRM of each corticosteroid. The signal intensities were found to be better in positive ionization mode when compared to the negative ionization mode because of better electrospray ionization of positively charged steroids. The mass transitions selected for Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone Triamcinolone acetonide and Imipramine were 431.2→413.13, 501.2→274.80, 361.20→343.20, 359.10→341.18, 393.10→373.11, 435→415.15 and 281.20→86.19 respectively. The MS/MS conditions for each transition were optimized in order to achieve the maximum signal-to-noise-ratio (S/N), and to avoid endogenous contamination of the chromatogram. The position of the spray needle was adjusted to achieve the optimal S/N for all compounds. Based on the physicochemical properties and compatibility with the mobile phase Imipramine was selected as internal standard. The analytes were extracted by solid phase extraction technique and chromatographed using ACE C-18 (35×4.6mm, i.d., 3µ) column. The mobile phase was methanol: 5mM ammonium acetate buffer pH 3.5 (90:10%v/v). 3µ column showed good peak shape when compared 4µ and 5µ columns. The auto sampler carryover was overcome by using 0.1% formic acid in water. No significant interferences were observed in the blank plasma samples of each analyte. Interferences were observed at the retention time of the analytes was found to be ≤9.26. A weighting factor of $1/x^2$ was used construct the calibration curve. The specificity/selectivity has been determined by using eight different sources of plasma including hemolysed and lipemic plasma.

The lower limit of quantification and the linearity range for each of the six drugs in the present study are tabulated below. The LLOQ levels achieved by the method reported here are lower than the methods available in the literature; depicting the sensitivity of the method discussed in this chapter.

Analyte	LLOQ	Range
Budesonide	10.000pg/ml	10.000-2000.000 pg/mL
Fluticasone -propionate	10.000pg/ml	10.000-1000.000 pg/mL
Prednisolone	1.000ng/mL	1.000 to 1000.000ng/mL
Prednisone	0.500ng/mL	0.500 to 500.000ng/mL
Dexamethasone	0.250ng/mL	0.250 to 250.000ng/mL
Triamcinolone acetonide	0.100ng/mL	0.100 to 1000.000ng/mL

The coefficient of determination (r^2) was ≥0.9943 for all the analytes indicating the method was linear over the range. For each analyte four sets of QC samples with six determinations at each level were analyzed in four different batches with linearity to determine intra batch precision and accuracy. Similarly four precision and accuracy batches were used to assess the inter-day precision and accuracy. Precision, expressed in terms percentage coefficient of variation(%CV) was found to be ≤ 6.70 for all the analytes from all the four batches. Plasma phospholipids are responsible for matrix effects in bioanalytical liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods and the method was optimized for the matrix effect. Internal standard normalized factor was calculated for each analyte at LQC and HQC levels using eight different sources of the plasma.

Stock solution stability and plasma stability of the analytes are very important for the validity of the method for pharmacokinetic and other clinical applications. The stability of analyte main stock and spiking stock solution at 2-8ºC and at room temperature was determined. The mean percentage change was found to be < 5.01 indicating the stock solution stability of the analytes. The integrity of study sample data can be ensured only if supporting stability data are available to confirm that degradation after sample collection has not occurred. This was tested at various stages of sample processing like bench top, freeze thaw process, wet extract, and dry extract, auto sampler at 5ºC and long term plasma storage stability at -70±5 º C. The results have shown that the mean percentage change was with the acceptable limits indicating the stability of the analyte at various sample processing stages. Thus the method was proven to be highly sensitive, selective, precise, accurate and rugged.

This developed and validated method can be extensively applied to pharmacokinetic studies involving all (Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone Triamcinolone acetonide) or some of the corticosteroids analyzed by this method.

Chapter-5

BIOANALYTICAL METHOD FOR DETERMINATION OF ETHINYL ESTRADIOL IN HUMAN PLASMA

Ethinyl estradiol (EE) is a potent synthetic estrogen that is widely used therapeutically with oral contraceptives (OC) primarily because of its high estrogenic activity. It is also used for the treatment of menopausal and post menopausal symptoms, treatment of female hypogonadism, osteoporosis, and as a palliative treatment in malignant neoplasms of breast and prostate [75]. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) enjoys wide spread popularity as the method of choice for bioanalysis of relatively polar and ionized compounds duetto its sensitivity and selectivity [82]. Literature has shown that the sensitivity of bioanalysis EE can be improved by derivatizating with dansyl chloride [115]. Low dose OC has been a growing concern about their possible interaction with co-administered drugs that induce the enzymes responsible for the metabolism of EE and result in failure of contraception in women using OC [76]. When a new OC formulation is developed, it is crucial to ensure optimum hormone exposure for guaranteeing the lowest dose to prevent pregnancy and avoid side effects. Therefore, a highly selective and sensitive bioanalytical method with a low limit of quantification (LLOQ) in pg/mL is essentially required to accurately measure ethinyl estadiol in human plasma samples.

5.1 Reference/Working standards

Ethinyl estradiol working standard was obtained from Famy Care(I) Limited , Mumbai, India and the internal standard ethinyl estradiol-d4 was obtained from Toronto Research Inc. Canada

5.2 Reagents and chemicals

Methanol, acetonitrile, ammonium formate, ammonium acetate, water, tertiary-butyl methyl - ether, sodium hydroxide ether, dansyl chloride, n-Hexane, ethyl acetate and formic acid were used in the method. The grade and manufacturers were same as mentioned in section- 4.2.

5.3 Instruments

The instruments used for method development and validation were Auto sampler, Analytical balance, Analytical Columns(Genesis,C-18, 100×4.6mm, 4µ, Chromolith,C-18, 50×4.6mm, 3µ),Column oven, Centrifuge, Deep Freezer, Hot air oven, Micro balance, Micropipette, MS Detector, Mulitipulse Vortexer, Nitrogen Evaporator, Solvent delivery module, Solid phase extraction unit, Ultrasonic bath, Vortexer and Water purification system. The Make/Model and manufacturer are same as that of section 4.3.

5.4 Blank plasma

Harvested K_2 EDTA blank plasma for method development and validation was obtained Navajeevan blood bank, Hyderabad, India and Blood Bank, Kasturba Hospital, Manipal, India.

5.5 Method Development

5.5.1 Scanning and Optimization of MS/MS detection parameters

Method development is initiated with scanning of the analyte solutions for the parent ions and its fragment ions using 100ng/mL solution of derivatized EE and derivatized EE-d4. Solution of analyte was injected using the syringe pump and scanned for the parent mass of the analyte. The parent ion was further scanned for product ions using MS/MS mode. EE-d4 was used as an internal standard. Mass scanning was done in the range of 50 to 600 m/z. The estrogenic steroid EE possesses the hydrophobic aromatic steroid core structure as well as the two hydroxyl functional groups common to estradiol. The non-polar nature and lack of ionizable function groups such as amine or carboxylic acid, steroids typically have very low sensitivity in electrospray ionization mass spectrometric analysis compared to polar and ionizable organic compounds. Atmospheric pressure chemical ionization (APCI) has showed less sensitivity over ESI for EE. ESI negative ionization mode exhibited very weak signals for the parent ion and weak product ions. Therefore chemical modification of EE to enhance the ESI-MS/MS sensitivity was undertaken by derivatizing with dansyl chloride [105]. The derivatization of phenolic groups of EE with dansyl chloride results in tertiary amine derivative, which gave a strong protonated molecule ($M+H^+$) using ESI-MS/MS under positive mode. A highly stable and intense product ion was formed at m/z 171, which corresponds to the neutral loss of EE-sulfate from the protonated derivative.

The fragment ion having high relative abundance was selected for selective reaction monitoring. EE was tuned manually in SRM mode with the mobile phase by using "T" which connects LC pump and syringe pump to the detector in order to optimize the detector parameters. Optimized ion source parameters are tabulated in the *table no.20*. Both Q1 and Q3 were operated under unit mass resolution. Protonated [$M+H^+$] ions were chosen as precursor ions for ESI-MS/MS studies. The SRM transitions monitored were 530 →171 for

EE and 534→ 171 for EE-d4. Derivatization procedure is shown in the *figure no.18*.The a result of mass scan is presented in the *figure 19(a) and (b)* for EE and EE-d4.

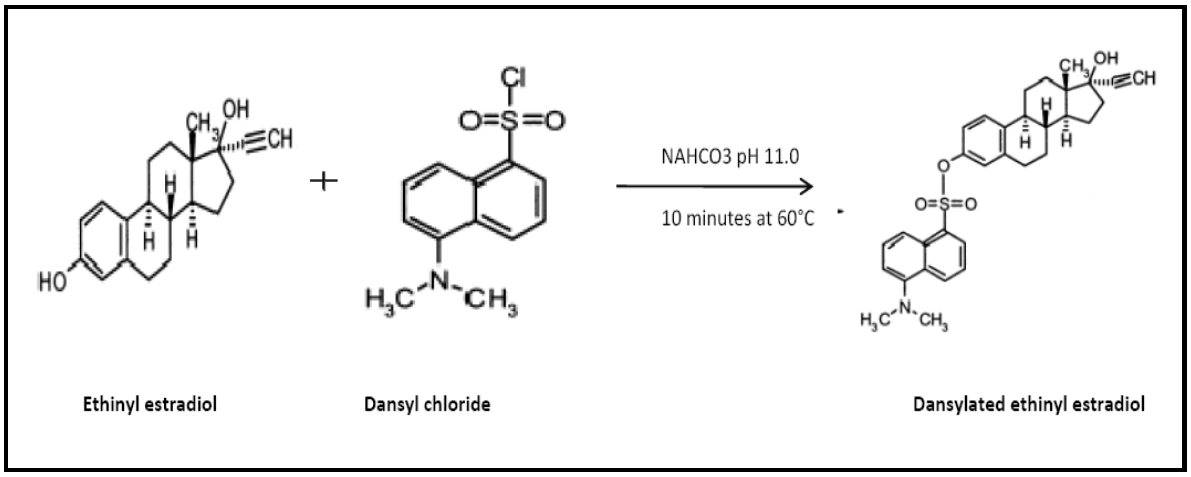

Figure No.18. Schematic diagram of derivatization procedure of ethinyl estradiol with dansyl chloride

Table No.20. Ion source parameters of ethinyl estradiol

Sr. No.	Ion source parameters	
01	Interface	ESI
02	Ionization mode	Positive
03	Spray voltage(V)	4500
04	Sheath gas pressure(arb)	40
05	Auxillary gas pressure(arb)	20
06	Capillary temperature(C)	350
07	Collision gas pressure(mTorr)	1.5
08	Chrom.filter	10
09	Skimmer offset	10
10	Collision Energy(eV)	29 for EE and 30 for d4-EE
11	Tube lens offset(V)	160 for EE and 150 for d4-EE
12	Skimmer offset(V)	17

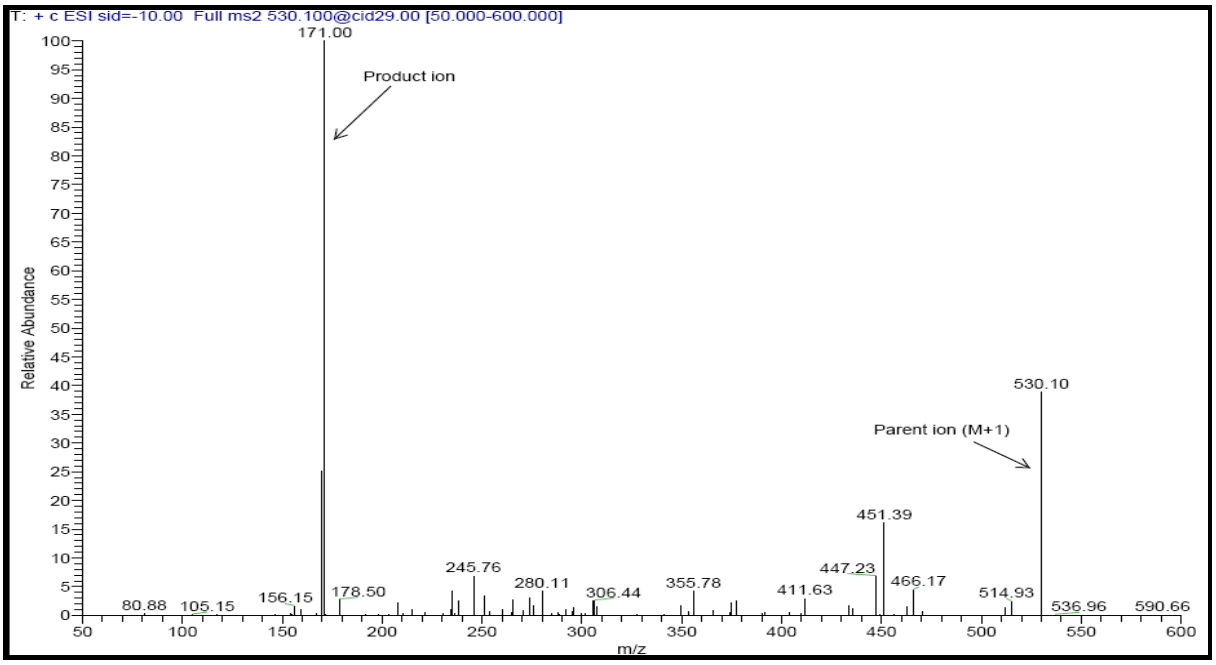

Figure No. 19(a). Parent and product scan analysis of Ethinyl estradiol

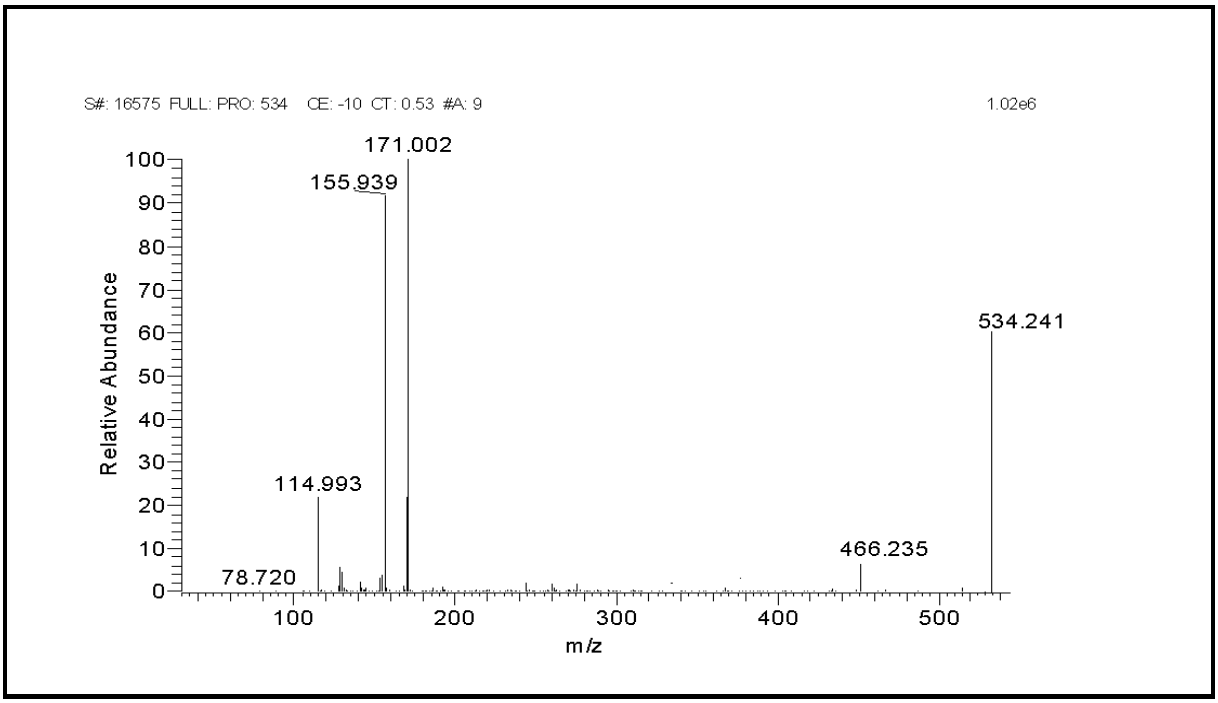

Figure No. 19(b). Parent and product scan analysis of Ethinyl estradiol-d4

5.5.2 Preparation of main stock solutions of ethinyl estradiol and ethinyl estradiol-d4

Ethinyl estradiol main stock of 1.000mg/mL solution was prepared by weighting 10.000 mg of ethinyl estradiol working standard into a 10.000mL volumetric flask and dissolved in 5.000mL of methanol and the volume was made up to the mark with methanol.

Ethinyl estradiol-d4 main stock of 0.200mg/mL solution was prepared by weighing 2.000mg ethinyl estradiol-d4 working standard into a 10.000mL volumetric flask and dissolved in 5.000mL of methanol and the volume was made up to the mark with methanol.

5.5.3 Preparation of intermediate stock solutions of ethinyl estradiol and internal standard.

Ethinyl estradiol intermediate stock solution (40.00ng/mL) was prepared by transferring 0.100ml of EE main stock solution was accurately transferred in to 10.000mL volumetric flask and volume was made up to 10.000mL with diluent. Vortexed to mix well. From this resulting solution 0.200mL of the solution was transferred into 50.000mL volumetric flask and volume made up to 50.00mL with the water: methanol (50:50%v/v).

Ethinyl estradiol-d4 internal standard working solution (10.000ng/mL) was prepared by transferring 0.005mL of main stock of EE-d4 into 100.00 volumetric flask and the volume was made up to 100.00mL with water: methanol (50:50%v/v).

5.5.4 Preparation of calibration spiking stock solutions of ethinyl estradiol

10.000mL of each spiking stock of Ethinyl estradiol was prepared as per the table mentioned below,

Calibration standard	Volume of intermediate stock taken(mL)	Spiking stock conc.(pg/mL)	Plasma conc.(pg/mL)
STD-1	0.010	40.00	2.00
STD-2	0.020	80.00	4.00
STD-3	0.125	500.00	25.00
STD-4	0.250	1000.00	50.00
STD-5	0.500	2000.00	100.00
STD-6	1.000	4000.00	200.00
STD-7	1.500	6000.00	300.00
STD-8	2.000	8000.00	400.00
STD-9	2.500	10000.00	500.00

5.5.5 Preparation Quality control spiking stock of ethinyl estradiol.

10.000mL of each spiking stock of Ethinyl estradiol was prepared as per the table mentioned below,

Quality Control	Volume of intermediate stock taken(mL)	Spiking stock conc.(pg/mL)	Plasma conc.(pg/mL)
LLOQ	0.010	40.00	2.00
LQC	0.030	120.00	6.00
MQC	1.250	5000.00	250.00
HQC	2.250	9000.00	450.00

5.5.6 Preparation of solutions and buffers

5.5.6.1 Preparation of buffer solutions

Weighed amount of the salt was transferred in to a glass beaker and 500.000mL of milli-Q/HPLC water was added and sonicated to mix well. The solution was then filtered through 0.22µm membrane filter. Different strengths of the buffer were prepared by weighing the salts as per the table shown in section 4.5.5.1, and 0.1% formic acid in water was prepares as per the procedure mentioned in section 4.5.5.2.

5.5.6.2 Preparation of 50mM sodium bicarbonate solution (pH 11)

0.420mg of sodium bicarbonate was weighed into a glass bottle and dissolved in 100.00mL of HPLC water and the pH was adjusted to 11 using dilute sodium hydroxide solution.

5.5.7 Optimizing chromatographic conditions

5.5.7.1 Effect of pH, stationary phases, solvent strength and flow rate.

The LC method was optimized in order to select suitable mobile phase with proper pH and column for achieving proper retention, peak shape, MS response and chromatographic resolution in presence of other plasma interferences. Mobile phases like Methanol: water, ACN: water, Methanol: ammonium acetate (2mM, 5mM, 10 mM), Methanol: Ammonium formate(5mM, 10mM), ACN: 0.1% formic acid in water, methanol: formic acid in water at the mobile phase composition of 90:10%v/v, 80:20%v/v and 70:30%v/v with Chromolith C18(50×4.6mm, 3µ) and Genesis C18(100×4.6mm, 4µ) columns. Methanol and 0.1% formic acid (90:10%v/v) in water was showed a good peak shape but response was insufficient to achieve the desired level of LLOQ. Finally an isocratic mode with Methanol: 5mM ammonium formate pH 3.0 (90:10%v/v) with the flow rate of 0.300mL/ min was selected

based on good peak shape, response and specific for the quantitation of EE from co-eluting endogenous plasma interferences. Column oven temperature was set at 40°C and auto sampler tray temperature was set at 5°C. Rinsing solution was optimized to Methanol: water (80:20%V/V) in order to remove the auto sampler carryover. The flush and wash volume was set 2000 µL/Sec. Injection volume was 25.0µL and overall chromatographic run time was 3.20 minutes.

Final chromatographic conditions:

Chromatographic mode	: Reversed Phase
Isocratic/gradient mode	: Isocratic
Internal Standard	: Ethinyl estradiol-d4
Rinsing solution	: Methanol: water (80:20%V/V)
Injection volume	: 25.0 µL
Column	: Chromolith speed rod, C-18, (50*4.6mm, i.d.,3µ)
Mobile phase	: Methanol:5mM ammonium formate pH3 (90:10 %v/v)
Column oven temperature	: 40°C
Auto sampler tray temperature	: 5°C
Flush volume	: 2000 µL/Sec
Wash volume	: 2000 µL/Sec
Flow rate	: 0.3 mL/min.
Run time	: 3.20mins
Retention time of ethinylestradiol	: 1.72 mins
Retention time of ethinyl estradiol-d4	: 1.72 mins

5.5.8 Optimization of extraction technique

Due to the non polar nature of the EE, liquid–liquid extraction could be ideal extraction technique. A simple liquid–liquid extraction procedure was developed to extract EE from plasma samples. The extraction procedure involves extraction of the analyte, derivatization and back extraction of the analyte[116]. In the first attempt different individual organic solvent

like tertiary butyl methyl ether (TBME), n-hexane and ethyl acetate were tried with and without buffering the plasma sample. Extracting with TBME showed good recovery but matrix effect was observed for the analytes. Acidification of the samples and subsequent extraction with TBME good recovery was observed, but significant matrix effect was observed. Ethyl acetate exhibited good recovery, but matrix effect was observed. n-hexane and TBME (65:30%v/v) has shown good recovery. The composition of n-hexane and TBME, derivatization time and temperature was optimized in order to achieve good recovery, and to eliminate endogenous estrogenic interferences. Summary of the first extraction trials are shown in the *figure no.20.*

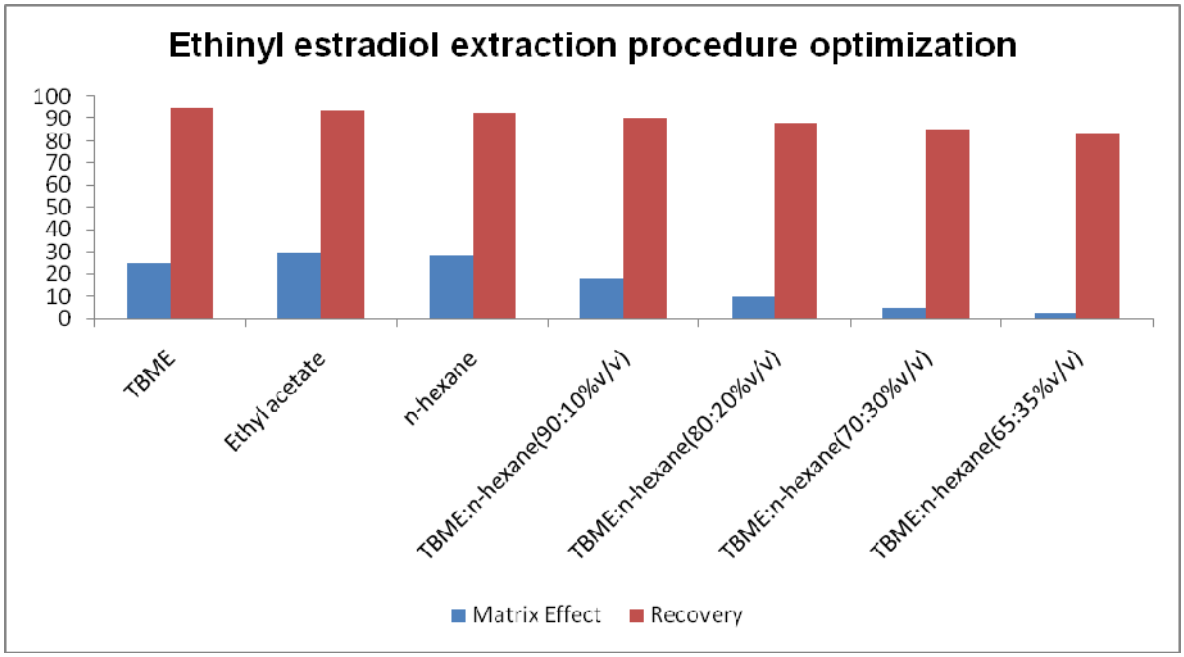

Figure No.20: Showing the extraction procedure optimization with different solvents for Ethinyl estradiol.

Final extraction Procedure: 0.760 mL of the blank sample and 40.00µL of analyte spiking stock were transferred to 10.00mL centrifuge tube and vortexed to mix. 40.00µL internal standard working solution (10.000ng/mL) was added to all samples except blank plasma where 40.0µL of diluent was added and vortexed to mix. 5.000mL of n-hexane: TBME (65:35% v/v) was added, and the tubes were capped and vortexed using mulitipulse vortexer at a speed of 80 units for 5 minutes. The samples were centrifuged at 3500 rpm for 5 min. The organic layer was separated and evaporated to dryness under a stream of nitrogen using

nitrogen evaporator at 40°C. To the residue, 0.100mL of sodium bicarbonate buffer (pH 11) was added and vortexed to mix. 0.200mL of dansyl chloride in acetone (1.00mg/mL) was then added. The tubes were vortexed to mix well and incubated at 60°C for 10 min in order to facilitate derivatization. Tubes were placed into another water bath to attain room temperature. Then 4.00mL of TBME was added and vortexed for 3 minutes, followed by centrifugation for 5 minutes at 3500 rpm at 4°C. Organic layer was separated and evaporated to dryness under the stream of nitrogen at 40°C. The residue was reconstituted with 0.4mL of methanol: 0.1% v/v formic acid (50:50%v/v). 25.0 µL of the reconstituted solution was injected into the LC–MS/MS system for analysis.

5.6 Method Validation

A high performance liquid chromatographic method with mass detection for the determination of ethinyl estradiol in human K_2EDTA plasma was developed as per the Guidance for Industry entitled 'Bioanalytical Method Validation' of the United States Food and Drug Administration, Center for Drug Evaluation and Research (CDER) May-2001[1].Ethinylestradiol and ethinylestradiol-d4 (Internal Standard) were extracted from an aliquot of human plasma using liquid-liquid extraction technique and injected in to a liquid chromatograph equipped with a tandem mass spectrometry detector. Quantitation was done by peak area ratio method. A weighted ($^1/x^2$) linear regression was performed to determine the concentration of analytes. All regressions and figures presented in this validation report were generated by LC-Quan software version 2.5.6. Validation was done as per the procedure and the acceptance criteria mentioned in Chapter-01.

5.6.1 Chromatography

A typical chromatogram obtained from blank sample (Processed blank K_2EDTA human plasma), lower limit of quantification and upper limit of quantification are represented in *Figure No. 21(a), (b), and (c)* respectively. The retention was 1.72 minutes for EE and EE-d4 respectively. The overall chromatographic run time is 3.20 minutes.

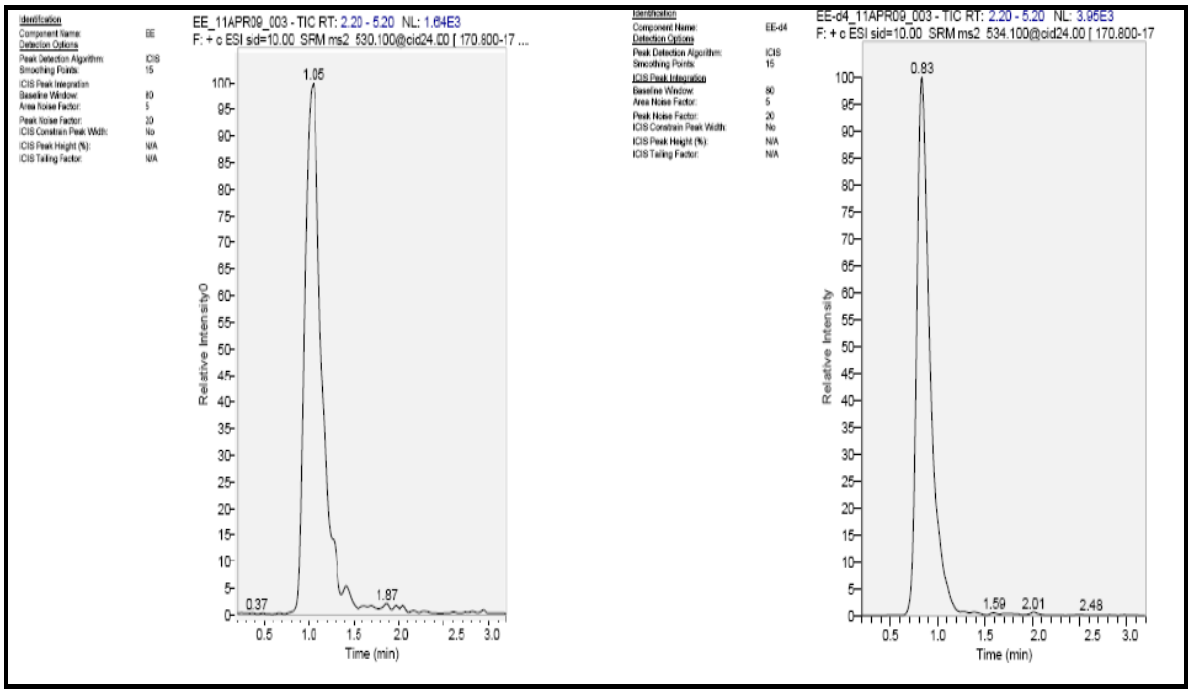

Figure No.21 (a). Representative chromatogram of blank plasma

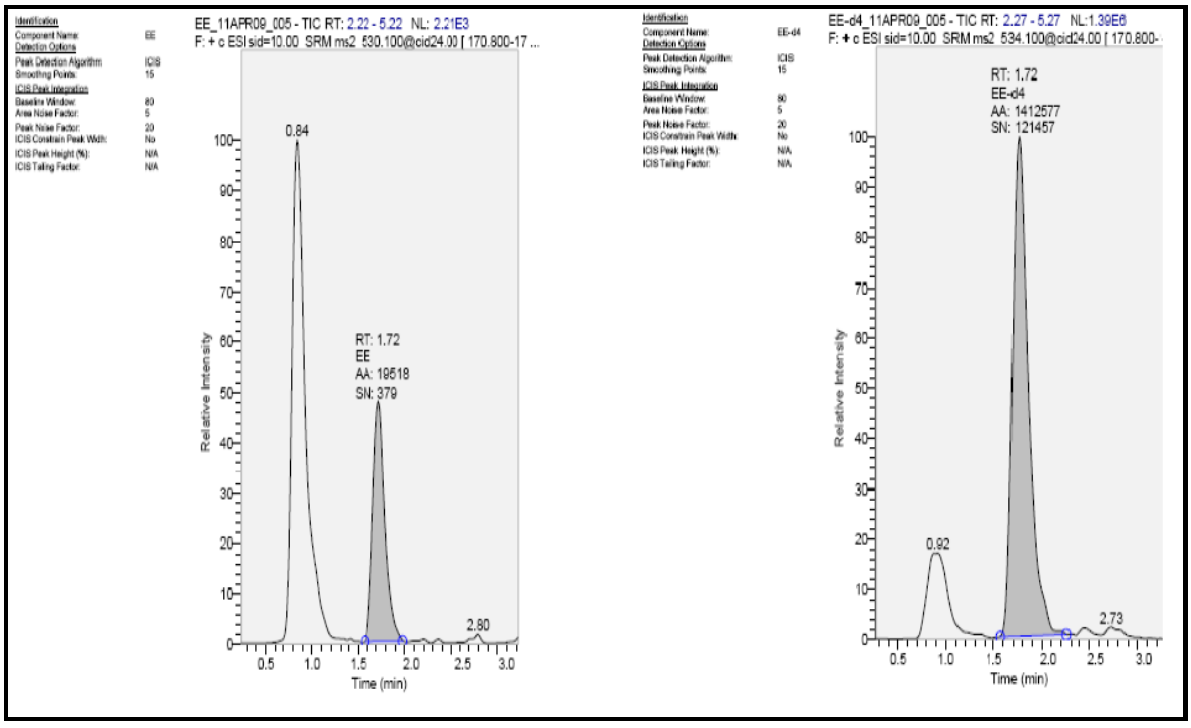

Figure No.21 (b). Representative chromatogram of ethinylestradiol LLOQ (2.000pg/mL)

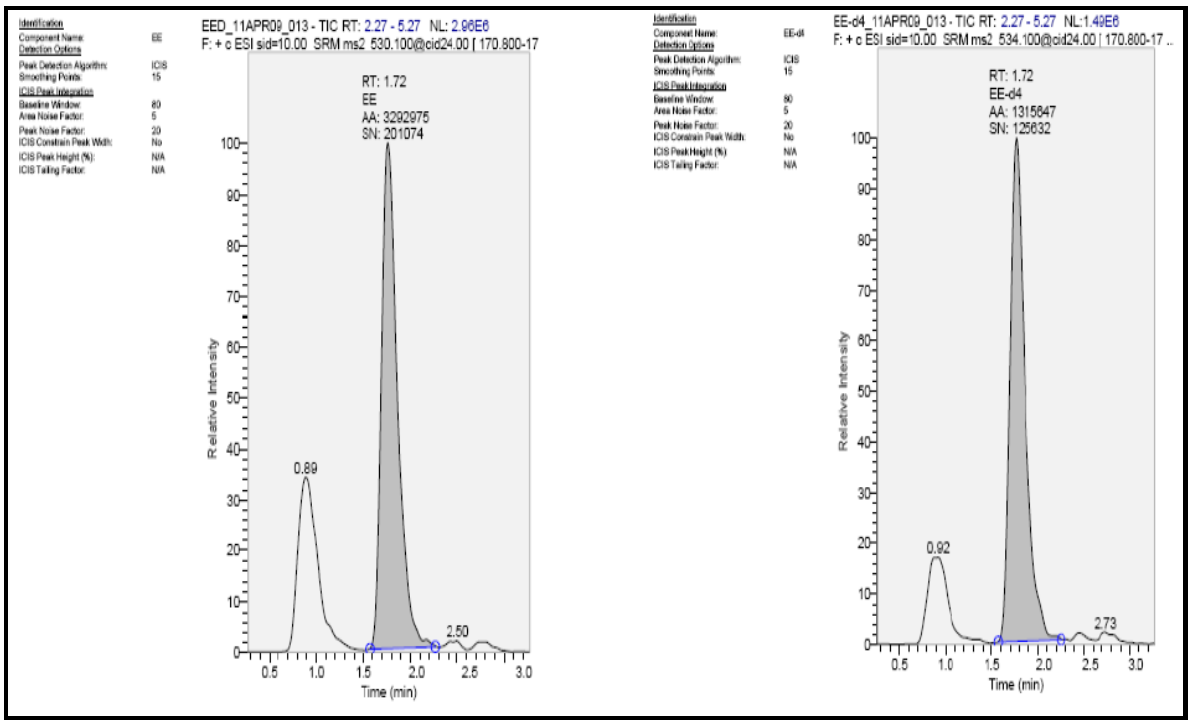

Figure No.21(c). Representative chromatogram of ethinylestradiol ULOQ (500.000pg/mL)

5.6.2 System Suitability

System suitability was performed during the beginning of every new sequence by injecting fine injections of aqueous equivalent MQC solution. The %CV for the peak response ratio of EE was found to be ≤ 0.74% and for its retention time was ≤ 0.37.The %CV for the retention time of EE-d4 was ≤0.22.

5.6.3 Specificity/Selectivity

Eight different lots of human K_2EDTA plasma including hemolysed and lipemic plasma were analyzed to determine the extent to which endogenous components in plasma which contributes to chromatographic interference with the analytes or internal standard. No significant interference from the blank plasma was observed at the retention time of analytes and internal standard. The data is presented in the *table no. 21.*

Table No.21 Specificity/selectivity of Ethinyl estradiol and Ethinyl estradiol-d4

SAMPLE NAME	Analyte Area	ISTD Area	SAMPLE NAME	Analyte Area	ISTD Area
EXT-LLOQ-LOT#K2E-01-01	18326	1235647	EXT-LLOQ-LOT#K2E-005	19697	1236545
EXT-LLOQ-LOT#K2E-01-02	18336	1123548	EXT-LLOQ-LOT#K2E-005	20354	1325475
EXT-LLOQ-LOT#K2E-01-03	18920	1236547	EXT-LLOQ-LOT#K2E-005	18706	1325478
Mean	18527	1198581	**Mean**	19586	1295833
BLANK SAMPLE-LOT#K2E-01	1058	11235	BLANK PLASMA LOT#K2E-005	1370	12302
% of Area	**5.71**	**0.94**	**% of area**	**6.99**	**0.95**
EXT-LLOQ-LOT#K2E-02-01	18476	1236547	EXT-LLOQ-LOT#K2E-006	21814	1325468
EXT-LLOQ-LOT#K2E-02-02	19862	1365876	EXT-LLOQ-LOT#K2E-006	18268	1236587
EXT-LLOQ-LOT#K2E-02-03	17964	1236585	EXT-LLOQ-LOT#K2E-006	18929	1156324
Mean	18767	1279669	**Mean**	19670	1239460
BLANK SAMPLE-LOT#K2E-02	1397	24563	BLANK SAMPLE-LOT#K2E-006	1193	48563
% of Area	**7.44**	**1.92**	**% of area**	**6.07**	**3.92**
EXT-LLOQ-LOT#K2E-03-01	18891	1236547	EXT-LLOQ-LOT#K2E-L-001	20098	1236587
EXT-LLOQ-LOT#K2E-03-02	19391	1365244	EXT-LLOQ-LOT#K2E-L-001	18696	1123654
EXT-LLOQ-LOT#K2E-03-03	19087	1263258	EXT-LLOQ-LOT#K2E-L-001	19062	1236548
Mean	19123	1288350	**Mean**	19285	1198930
BLANK SAMPLE-LOT#K2E-03	2614	22365	BLANK SAMPLE-LOT#K2E-L-001	988	15324
% of Area	**13.67**	**1.74**	**% of area**	**5.12**	**1.28**
EXT-LLOQ-LOT#K2E-04-01	23349	1325487	EXT-LLOQ-LOT#K2EH-001	13999	1325476
EXT-LLOQ-LOT#K2E-04-02	21954	1236547	EXT-LLOQ-LOT#K2EH-001	18952	1236547
EXT-LLOQ-LOT#K2E-04-03	17071	1136524	EXT-LLOQ-LOT#K2EH-001	18889	1236584
Mean	20791	1232853	**Mean**	17280	1266202
BLANK SAMPLE-LOT#L-004	1126		BLANK SAMPLE-LOT#K2EH-001	1962	13254
% of area	**5.42**		**% of area**	**11.35**	**1.05**

5.6.4 Carry Over Check

Carryover check of the sample was carried out by injecting the highest concentration of the calibration curve (ULOQ) and internal standard followed by the reconstitute solution and extracted blank plasma. No significant carryover was observed in reconstitution solution and extracted blank plasma.

5.6.5 Sensitivity

The lower limit of quantification was 2.000pg/mL with a % coefficient of variation being 4.21% and mean % nominal concentration of 93.60%. Signal to noise ratio was ≥ 192.

5.6.6 Calibration curve

Calibration curves were found to be over a calibration range of 2.000 to 500.000 pg/mL. The calibration model was determined by testing the algorithms linear/quadratic, 1/x weighted linear/quadratic, $1/x^2$ weighted linear/quadratic. The calibration model of $1/x^2$ weighted linear regression gave a good fit and the model was reproducible that minimizes the bias of the back-calculated values. The coefficient of determination was found to be ≥0.9976. The data of the back calculated values are presented in *Table No 22*. A typical calibration curve is presented in *figure no.22.*

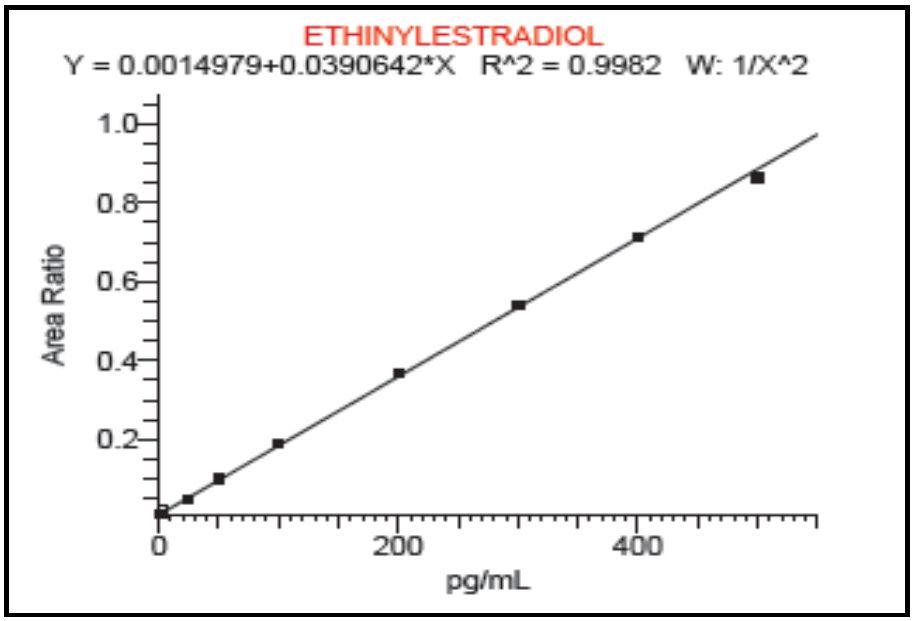

Figure No. 22. Representative calibration curve of Ethinyl estradiol

Table No.22. Back calculated concentrations of Ethinyl estradiol calibration standards

Linearity	Concentration(pg/mL)								
	STD-1	**STD-2**	**STD-3**	**STD-4**	**STD-5**	**STD-6**	**STD-7**	**STD-8**	**STD-9**
	2.000	4.000	25.000	50.000	100.000	200.000	300.000	400.000	500.000
Mean	2.027	4.368	26.002	53.780	103.680	209.819	312.252	404.443	506.455
SD	0.136	0.298	0.558	1.677	3.375	4.903	8.073	11.071	9.642
%CV	6.71	6.82	2.15	3.12	3.26	2.34	2.59	2.74	1.90
Mean % nominal Conc.	101.35	109.20	104.01	107.56	103.68	104.91	104.08	101.11	101.29

5.6.7 Precision and Accuracy (P&A)

Within-batch or intra-batch accuracy and precision evaluation were assessed by analyzing one calibration curve and 4 sets of QC samples (6 replicates each of the LLOQC, LQC, MQC and HQC) in four different batches. Mean percentage nominal concentration at LLOQC for Batch-01, Batch-02, Batch-03 and Batch-04 were 99.15%, 92.85%, 101.44% and 102.23% respectively. Percentage coefficient of variation at LLOQC for Batch-01, Batch-02, Batch-03 and Batch-04 were 6.73%, 7.02%, 4.25% and 6.53% respectively. Mean percentage nominal concentration at LQC, MQC and HQC for Batch-01, Batch-02, Batch-03 and Batch-04 were 102.86 to 104.48%, 98.23 to 103.79%, 104.77 to 109.97% and 100.26 to 105.87% respectively. Percentage coefficient of variation at LQC MQC and HQC for Batch-01, Batch-02, Batch-03 and Batch-04 were ≤4.04%, ≤5.03%, ≤4.58% and ≤2.42% respectively. Between batch or inter-batch results have shown mean percentage nominal concentration at LLOQC was 98.52 and at LQC, MQC and HQC was 100.55 to 104.10. Percentage coefficient of variation at LLOQC was 6.65 and at LQC MQC and HQC was for Batch-01, Batch-02, Batch-03 and Batch-04 were ≤3.33%.

5.6.8 Recovery

The percentage recovery of EE and EE-d4 was determined by comparing the mean peak area of extracted LQC, MQC and HQC samples with freshly prepared post spiked (unextracted) LQC, MQC and HQC samples respectively. Mean % recovery at LQC, MQC and HQC were found to be 84.89%, 88.50% and 78, 58% respectively. Mean % recovery of EE across QC levels was 83.99% and variability across QC level was 5.98%. The mean percentage recovery for EE-d4 was 84.09.

5.6.9 Stability

5.6.9.1 Stock solution stability

EE and EE-d4 stock solutions were prepared and aliquots of stocks were stored at 2-8°C (stability sample). After 15 days EE MQC spiking stock and EE-d4 working solution was prepared from the main stock (stability samples) and analyzed with the freshly prepared aqueous equivalent MQC spiking stock and working solution of ISTD. EE, EE-d4 main stock

solution and EE spiking stock at 2-8ºC was found to be stable for 15 days. Main stock and spiking stock at room temperature for 12.00hrs was found to be stable. The results were presented in the *table no. 23.*

Table No. 23. Stock solution stability of Ethinyl estradiol and Ethinyl estradiol-d4

Stock solution Stability	**Mean area of comparison sample**	**Mean area of Stability sample**	**Mean % Change**
Main stock solution stability for 15 days at 2-8ºC	1654872	1716435	3.72
Spiking stock solution stability for 15 days at 2-8ºC	1654872	1612502	-3.56
Main stock solution stability for 12 hrs at room temp.	1654872	1633651	-1.28
Spiking stock solution stability for 12 hrs at room temp.	1654872	1636054	-1.14

5.6.10 Stability of drug in plasma

The procedure and acceptance criteria for all stability parameters were same as mentioned in the section 4.6.9.2. Bench top (BT) stability for 8 hrs, in-injector stability for 58 hrs at 5°C, Freeze thaw (FT) stability for 4 cycles, dry extract (DE) stability for 4 hrs at room temperature, wet extract (WE) stability for 6 hrs at room temperature, process stability of the samples has for 4 hrs at room temperature and long term (LT) stability for 200 days has been performed.. The results of all stability experiments were found to be within the acceptance criteria, thus proving the stability of the Ethinyl estradiol in plasma. The results were presented in the *table No. 24.*

Table No.24: Plasma stability of Ethinyl estradiol

Analytes	BT-Stability	In-injector stability for	FT Stability	DE stability	WE stability	Process stability	LT stability
Mean % change							
LQC	-2.40	-1.28	-7.40	-4.58	-1.36	-1.52	-5.06
HQC	-2.83	-1.14.	-4.40	-6.94	-1.92	-1.13	-6.02

5.6.11 Dilution integrity

Dilution integrity experiment was carried out at six replicates of two times (½ dilution) and of four times diluted (¼ dilution) 2 X ULOQ samples. The samples were prepared and

concentrations were calculated including the dilution factor against the calibration curve. The mean percentage nominal concentration of EE was found to be 93.66% and 89.54% for ¼ and ½ dilution. Percentage coefficient of variation was ≤ 9.65%.

5.6.12 Matrix effect

Matrix effect was carried out at LQC and HQC levels in six different lots of K_2EDTA plasma including hemolysed and lipemic plasma. Drug free K_2EDTA plasma was processed and the extracted matrix was post spiked with LQC and HQC spiking stock solutions and internal standard. The aqueous equivalent LQC and HQC was also injected and analyzed. Matrix factor (ion suppression/ enhancement) was determined by comparing the area response of post spiked LQC, HQC with aqueous equivalent LQC and HQC. No significant effect was observed. The % CV across different lots of ISTD normalized matrix factor at LQC and HQC is 2.59 % and 2.08% respectively.

5.6.13 Hemolysis and lipemic effect

The procedure and the acceptance criteria for these validation parameters are same as mentioned in section 4.6.12. Mean Percentage nominal concentration of EE for hemolised samples at LQC and HQC were 99.35 % and 104.38% respectively and percentage coefficient of variation of EE was ≤ 4.81%. Mean Percentage nominal concentration of EE for lipemic samples at LQC and HQC were 99.35 % and 104.38% respectively and percentage coefficient of variation of EE was ≤ 4.81%.

5.6.14 Ruggedness

Ruggedness of the method was determined by analyzing the two batches of P&A samples processed by two different analysts. One P&A analyzed using a different lot of the column and another P&A prepared from different lots of solvents. The mean percentage nominal concentration and the coefficient of variation across these batches were calculated. Mean percentage nominal concentration at LLOQC level was found to be 104.24% and at LQC, MQC and HQC were found to be 107.69 to 105.87%. %CV at LLOQC level was 8.56% and at LQC, MQC &HQC was ≤ 2.36. EE was found to be stable with change in external variables like analysts, change in make of the solvents and change in lot number of the column.

5.6.15 Production batch run (Precision and Accuracy).

A production batch comprising of 160 samples, which includes linearity and randomly placed QC's were analyzed. This run was planned in order to simulate the number of samples that could be acquired in a production batch, during subject sample analysis. Mean % nominal concentration was found to be 98.56 to 103.09% and the percentage coefficient of variation was found to be ≤ 3.14.

5.6.16 Reinjection Reproducibility

After the completion of analysis of one of the P&A batch, the calibration standards, LQC and HQC samples were retained in auto sampler and were re-injected. The percentage differences between mean calculated concentrations of QCs were calculated between two batches at each level. The % difference between two batches at LQC and HQC were -4.65% and HQC -0.83% respectively.

5.7 Conclusion

Ethinyl estradiol (EE) is a potent synthetic estrogen that is widely used therapeutically, mainly as oral contraceptives. Low plasma levels of ethinyl estradiol result in menstrual bleeding and unanticipated pregnancies while high levels of EE result in hypertension and vascular disease. Therefore it is essential to monitor the therapeutic levels of EE. Methods have been reported to determine EE in biological fluids. But these methods are not sensitive enough to measure the EE in pg/mL levels [106]

Present work describes a simple, rapid and rugged HPLC–MS/MS method for the determination of ethinyl estradiol in human K_2EDTA plasma using ethinyl estradiol-d4 (EE-d4) as the internal standard (IS). The method was validated in accordance with USFDA guidelines "Guidance for the Industry "Bioanalytical Method Validation, 2001" [1]. The mass spectra for EE and EE-d4 were obtained by scanning derivatized ethinyl estradiol and ethinyl estradiol-d4 (IS). The derivatization was done by using dansyl chloride in order to increase the sensitivity of the analytes. Scanning with positive ionization mode exhibited high signal intensities when compared to the negative ionization mode. The most abundant and stable product ions were selected for selective reaction monitoring (SRM) of the analytes. The mass transitions of 530→171 and 534→171 were selected for EE and EE-d4 respectively. The MS/MS parameters were optimized by direct infusion of the analytes through the mobile

phase in to mass spectrophotometer source in order to achieve the maximum response. The position of the spray needle was adjusted to achieve the optimal response. The analytes were extracted from the plasma by liquid-liquid extraction and derivatized with dansyl chloride prior to analysis[116]. Analytes were chromatographed using Chromolith, C-18, (50×4.6mm, i.d.,3µ) column. The mobile phase was methanol: 5mM ammonium formate pH 3. (90:10%v/v) with a flow rate of 0.300mL/min. the detection conditions were optimized by pH adjustment of the mobile phase. The auto sampler carryover was eliminated by using Methanol: water (80:20%v/v). No significant interferences were observed in the blank plasma samples of each analyte. $1/x^2$ weighted regressions generated by using peak area ratio v/s concentration to estimate the analyte concentrations. The specificity/selectivity has been determined using eight different sources of plasma including hemolysed and lipemic plasma. The coefficient of determination (r^2) was found to be ≥0.9976. Linearity range of the method was 2.000-500.000pg/mL. The lowest concentration quantified was 2.000pg/ml with satisfacory accuracy and precision. The intra-day precision ranged from 2.42 to 7.02%, while inter-day precisions ranged between 3.30 to 6.65%. The intra-day accuracies ranged from 92.85 to 109.97%, while the inter-day accuracies ranged from 98.52 to 104.10%. The method was developed and validated to achieve very low levels of plasma concentration (2.000Pg/mL) and the method was also successfully applied for the pharmacokinetic study. Due to high sensitivity and short analysis run time this method can be applied to any type of clinical studies.

Chapter-6

BIOANALYTICAL METHOD FOR DETERMINATION OF LEVONORGESTREL IN HUMAN PLASMA

Levonorgestrel (LEV) is a synthetic female contraceptive (OC) hormone used in pregnancy prevention. Proper monitoring of plasma levels is required to bring changes in formulations by finding effective dose and thus limiting clinical side effects. The reported methods are not sensitive enough for pharmacokinetic analysis when Levonorgestrel is given in low doses. Reported HPLC methods are not sensitive enough to quantify Levonorgestrel in pharmacokinetic analysis of low doses [89]. Cytochrome P450 enzyme inducers have been reported to increase the incidence of breakthrough bleeding and unwanted pregnancies in women using OC containing levonorgesstrel. Thus, it is very important to explore the potential interaction of new drug candidates with low dose OC during drug development process to ensure the optimum OC exposure being maintained during the concomitant therapy. To meet these needs, a highly sensitive analytical method with a low limit of quantification (LLOQ) level for LEV is required to accurately measure analyte concentrations in human plasma samples.

6.1 Reference/Working standards

Dexamethasone reference standard was purchased was of US Pharmacopeia and
Levonorgestrel working standard was obtained from the Famy care Ltd, Mumbai India.

6.2 Reagents and chemicals

Methanol, acetonitrile, ammonium formate, ammonium acetate, water, n-Hexane, ethyl-acetate, perchloric acid and hydrochloric acid were used in the method. The grade and manufacturers were same as mentioned in section- 4.2.

6.3 Instruments

The instruments used for method development and validation were Auto sampler, Analytical balance, Analytical Columns(Genesis,C-18, 100×4.6mm, 4µ, ACE, C-18, 35×4.6mm, 3µ, Kromacil,C-18, 50×4.6mm, 4µ and Hypersil gold,C-18, 50×4.6mm,4µ),Column oven, Centrifuge, Deep Freezer, Hot air oven, Micro balance, Micropipette, MS Detector, Mulitipulse Vortexer, Nitrogen Evaporator, Solvent delivery module, Ultrasonic bath, Vortexer and Water purification system. The Make/Model and manufacturer are same as that of section 4.3.

6.4 Blank plasma

Harvested K_2 EDTA blank plasma for method development and validation was obtained Navajeevan blood bank, Hyderabad, India and Blood Bank, Kasturba Hospital, Manipal, India.

6.5 Method Development

6.5.1 Scanning and Optimization of MS/MS detection parameters

Mass spectrum analysis was initiated with electrospray ionization (ESI). 100.00ng/ml solution of Levonorgestrel in methanol was directly infused in to source interface at the flow rate of 10µL/min through infusion syringe pump. The spectra were recorded from 50 to 600m/z using atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) interface. Atmospheric pressure chemical ionization (APCI) in positive mode showed highly stable signals but the intensity was low when compared to ESI. High relative abundance for the parent and product ions was observed with positive ESI. MS conditions like spray voltage, collision gas pressure, auxiliary gas pressure, sheath gas pressure, collision energy, tube lens offset voltage, skimmer offset voltage were optimized to have a high MS response. Dexamethasone was used as an internal standard. Optimized ion source parameters are tabulated in *Table No. 25.* Both Q1 and Q3 were operated under unit mass resolution (0.7 Da at full width half-maximum) maintained at unit mass resolution. The most protonated molecular ions $[M+H^+]$ *m/z* at 313.0 and 393.0 to the product ions *m/z* at 109 for LEV and 293 for DEX (used as internal standard) were observed and used for quantitative SRM. The results of mass scan of LEV and DEX were presented in *figure no.23 (a) and 23(b)*.

Table No.25. Ion source parameters for Levonorgestrel and Dexamethasone

Sr. No.	Ion source parameters	
1	Interface	ESI
2	Ionization mode	Positive
3	Spray voltage(V)	4500
4	Sheath gas pressure(arb)	40
5	Auxillary gas pressure(arb)	20
6	Capillary temperature(C)	350
7	Collision gas pressure(mTorr)	1.5
8	Chrom.filter	10
9	Skimmer offset	10
10	Collision Evergy(eV)	44 for LN and 9 for DEX
11	Tube lens offset(V)	96 for LN and 100 for DEX

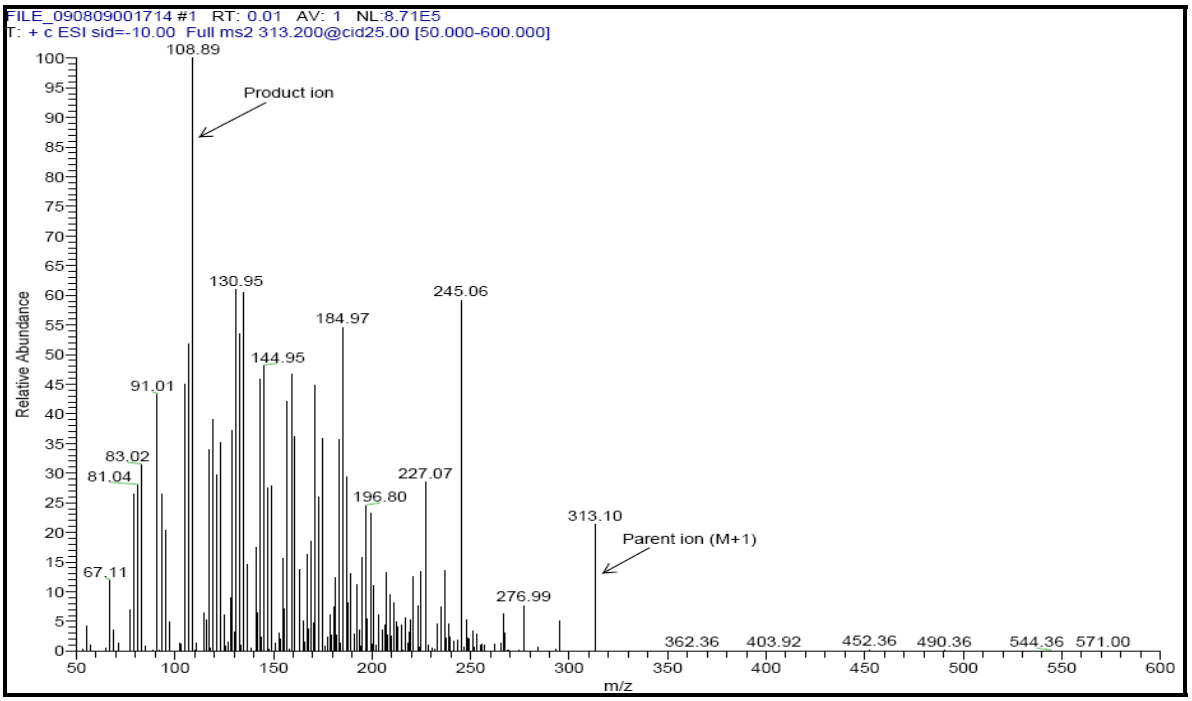

Figure No. 23(a). Parent and product scan analysis of Levonorgestrel

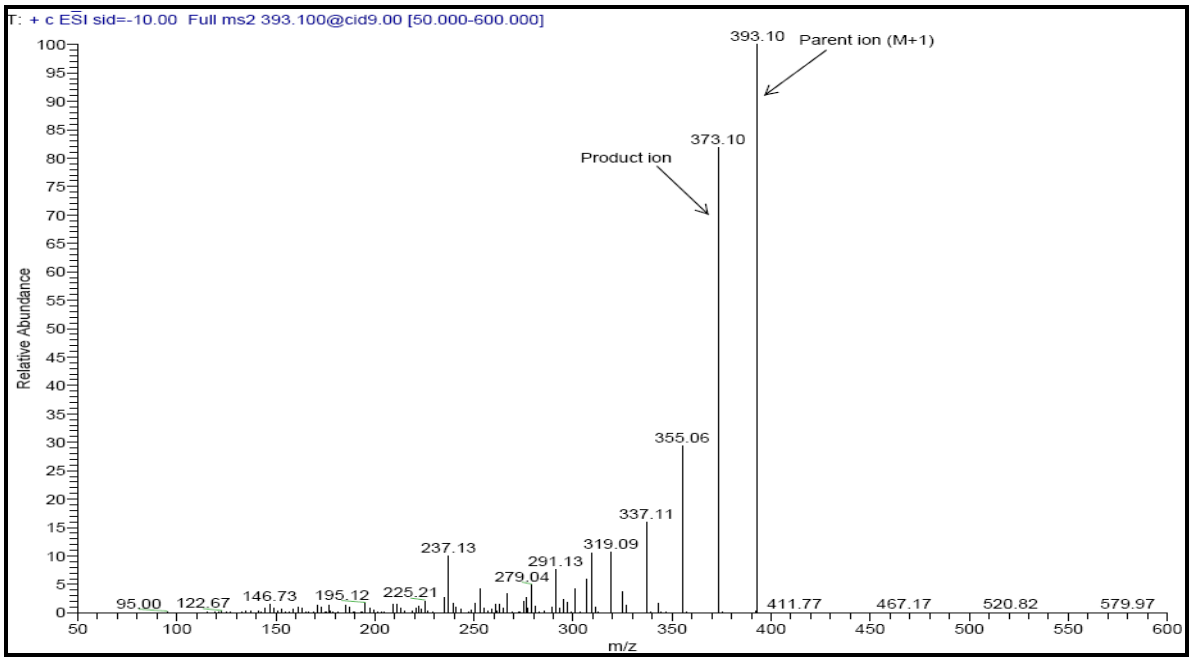

Figure No. 23(b). Parent and product scan analysis of Dexamethasone

6.5.2 Preparation of main stock solutions of Levonorgestrel and Dexamethasone.

Levonorgestrel main stock of 1.000mg/mL solution was prepared by weighing the working standard equivalent to10.000 mg into 10.000mL volumetric flask, dissolved with 5.000mL of methanol and volume made up to the mark with methanol.

Dexamethasone main stock of 1.000mg/mL solution was prepared by weighing the reference standard equivalent to 10.000 mg into 10.000mL volumetric flask, dissolved with 5.000mL of methanol and volume was made up to the mark with methanol.

6.5.3 Preparation of intermediate stock solutions of Levonorgestrel and Dexamethasone working solution.

Levonorgestrel intermediate stock solution of 100.00ng/mL was prepared by transferring 0.010ml of the main stock solution was transferred into a 10.000mL volumetric flask and volume was made up to 10.000mL with methanol: water (50:50%v/v) and vortexed to mix well. From this resulting solution 0.500mL of the solution was transferred into 50.000mL volumetric flask and volume made up to 50.000mL with the methanol: water (50:50%v/v).Dexamethasone internal standard working stock solution (200.000ng/mL) was

prepared by transferring 0.020ml of the Dexamethasone main stock solution in to 100.000mL volumetric flask and volume was made up to mark with methanol: water (50:50%v/v).

6.5.4 Preparation of calibration standards spiking stock solution of Levonorgestrel

10.000mL of each spiking stock of Levonorgestrel was prepared as per the table mentioned below,

Calibration standard	Volume of intermediate stock taken	Spiking stock conc. (ng/mL)	Plasma conc. (ng/mL)
STD-1	0.002	2.00	0.10
STD-2	0.004	4.00	0.20
STD-3	0.020	20.00	1.00
STD-4	0.200	200.00	10.00
STD-5	0.800	800.00	40.00
STD-6	1.600	1600.00	80.00
STD-7	2.400	2400.00	120.00
STD-8	3.200	3200.00	160.00
STD-9	4.000	4000.00	200.00

6.5.5 Quality control samples spiking stock preparation

10.000mL of each spiking stock of Levonorgestrel was prepared as per the table mentioned below,

Quality Control	Volume of intermediate stock taken	Spiking stock conc. (ng/mL)	Plasma conc. (ng/mL)
LLOQ	0.002	2.00	0.10
LQC	0.006	12.00	0.60
MQC	2.000	2000.00	100.00
HQC	3.600	3600.00	180.00

6.5.6 Preparation of solutions and buffers

Weighed amount of the salt was transferred in to a glass beaker and 500.000mL of milli-Q/HPLC water was added and sonicated to mix well. The solution was then filtered through 0.22µm membrane filter. Different strengths of the buffer were prepared by weighing the salts as per the table shown in section 4.5.5.1, and 0.1% formic acid in water was prepared as per the procedure mentioned in section 4.5.5.2.

6.5.7 Optimizing chromatographic conditions

6.5.7.1 Effect of pH, stationary phases, solvent strength and flow rate

Liquid Chromatographic method was optimized in order to select a suitable mobile phase with proper pH and column for achieving proper retention, peak shape, MS response and chromatographic resolution with other plasma interferences. Mobile phases like Methanol: water, ACN: water, methanol: ammonium acetate (2mM, 5mM, 10mM), Methanol: ammonium formate (5mM, 10mM), ACN: 0.1% formic acid in water, methanol: formic acid in water at different mobile phase composition like 90:10%v/v,80:20v/v, 70:30v/v were studied. Different C18 columns like Genesis (100×4.6mm, i.d., 4µ, 50×4.6mm, 4µ), ACE (35×4.6mm, i.d., 3µ), Kromacil (50×4.6mm,i.d., 4µ) and Hypersil gold (50×4.6mm, i.d.,4µ) columns were also investigated. Best results were obtained with Hypersil gold (50×4.6mm, i.d.,4µ) column with the mobile phase composition of methanol and 0.1% formic acid in water(87:13% v/v). Genesis C18 (100×4.6mm, i.d., 4µ) column showed long run time but peak shape and response was found to be good. With ammonium acetate and ammonium formate buffers response was low and interferences were observed with the blank sample at the retention time of analytes. With Hypersil gold, C18 (50×4.6mm, i.d., 4µ) column different flow rates were tested and a flow rate of 0.400mL/ min gave an optimum response. Column oven temperature was set at 40°C and auto sampler tray temperature was set at 5°C. Rinsing solution was optimized to 0.1% formic acid in [(Methanol: water (80:20%v/v)] in order to eliminate the auto sampler carryover. The flush and wash volume was set at 2000 µL/Sec. Injection volume was 25.0µL and overall chromatographic run time was 1.60 min.

6.5.7.2 Selection of internal standard

The internal standard was selected on the basis of chemical structure, polarity and solubility characteristics. Based on the physicochemical properties Dexamethasone was selected and chromatographed. The response of the Dexamethasone was found to be reproducible with the optimized mobile phase conditions.

6.5.7.3 Final chromatographic conditions

Chromatographic mode : Reversed Phase

Isocratic/gradient mode : Isocratic

Internal Standard	: Dexamethasone
Rinsing solution	: 0.1% formic acid in (methanol: water (80:20% v/v)
Injection volume	: 25.0 µL
Column	: Hypersil Gold, C-18, 50*4.6mm, 5µ
Mobile phase	: Methanol: 0.1% formic acid in water (87:13% v/v)
Column oven temperature	: 40°C
Auto sampler tray temp.	: 5°C
Flush volume	: 2000 µL/Sec
Wash volume	: 2000 µL/Sec
Flow rate	: 0.400mL/min.
Run time	: 1.60 mins
Retention time of Levonorgestrel	: 1.29 mins
Retention time of Dexamethasone	: 1.18 mins

6.5.7.4 Selection of extraction technique

Due to weak polarity extraction of Levonorgestrel from plasma is a challenging task. Protein precipitation extraction technique with acetonitrile and methanol were tried but showed very low recovery. Precipitation with perchloric acid and hydrochloric acid demonstrated matrix effect and low recovery. Due to the non polar nature of Levonorgestrel liquid-liquid extraction was tried with neat organic solvents ethyl acetate and n-hexane. Extracting with ethyl acetate showed good recovery but about 25% matrix effect was observed for the analyte. Samples extracted with n-hexane have less recovery and no matrix effect. Looking into the inefficiency of the liquid-liquid extraction of the neat organic solvents, various combinations of ethyl acetate and n- Hexane like 70:30%v/v, 820:20%v/v and 90:10% v/v were evaluated for the recovery and matrix effect. The optimized extractant was of ethyl acetate and n-hexane (80:20%v/v). Samples extracted with the combination of the solvents exhibited good recovery along with absence of matrix effect.

Final Extraction Procedure: 0.015mL of analyte and 0.275 mL plasma samples were transferred into a 10.00 mL centrifuge tubes vortexed to mix. 0.015mL of internal standard

working solution (200.00ng/mL) was added and vortexed to mix. 2.00 ml n-hexane: ethyl acetate mixture (20:80% v/v) was added and then vortexed for 5 min. The samples were then centrifuged for 5 min at 3500rpm. The organic layer was separated and evaporated to dryness under a stream of nitrogen at 40 °C. The residue was reconstituted with 400µl mobile phase and vortexed for 30 sec. 25µL of this solution was injected into LC-MS system.

6.6 Method Validation

A high performance liquid chromatographic method with mass detection for the determination of Levonorgestrel in human K_2EDTA plasma was developed as per the Guidance for Industry entitled 'Bioanalytical Method Validation' of the United States Food and Drug Administration, Center for Drug Evaluation and Research (CDER) May-2001[1]

Levonorgestrel and Dexamethasone (Internal Standard) were extracted from an aliquot of human plasma using liquid-liquid extraction technique and injected in to a liquid chromatograph equipped with a tandem mass spectrometry detector. Quantitation was done by peak area ratio method. A weighted ($^1/x^2$) linear regression was performed to determine the concentration of analytes. All regressions and figures presented in this validation report were generated by LC-Quan software version 2.5.6. Procedure and acceptance criteria of validation parameters were same as that of chapter-4.

6.6.1 Chromatography

A typical chromatogram obtained from blank sample (Processed blank K_2EDTA human plasma), lower limit of quantification and upper limit of quantification are represented in *figure no. 24(a), (b) and (c)* respectively. The retention was 1.17 and 1.29mins for LEV and DEX respectively. The overall chromatographic run time is 1.60 minutes.

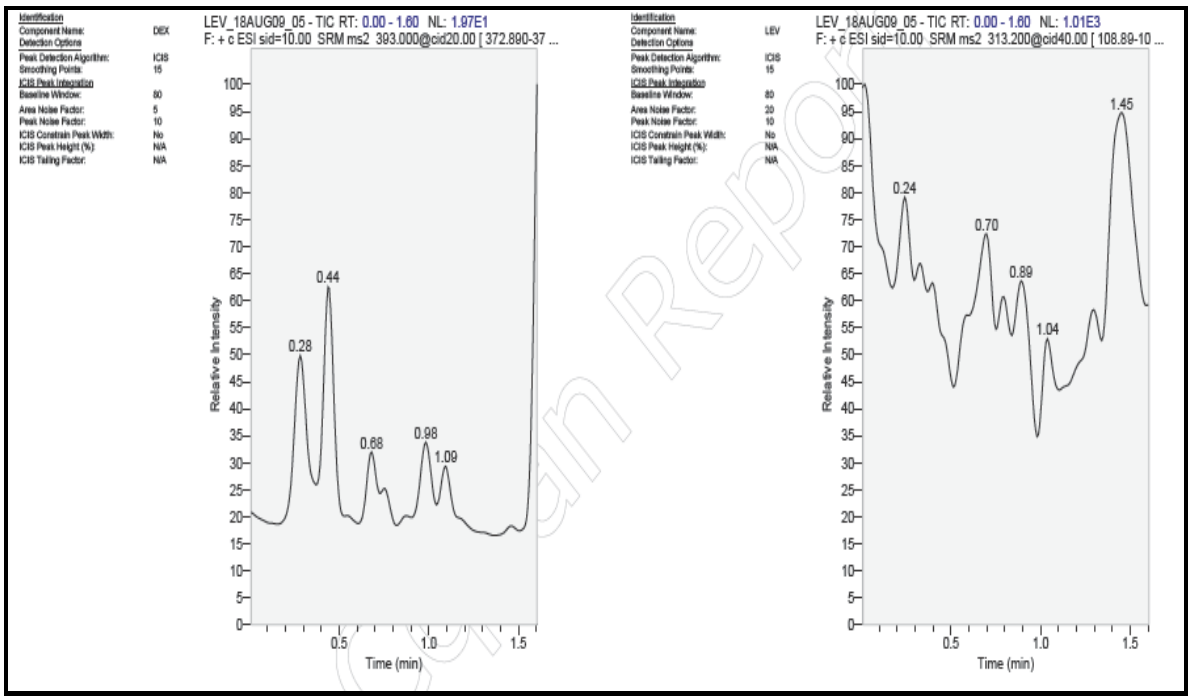

Figure No.24(a). Representative chromatogram of Levonorgestrel and Dexamethasone blank plasma

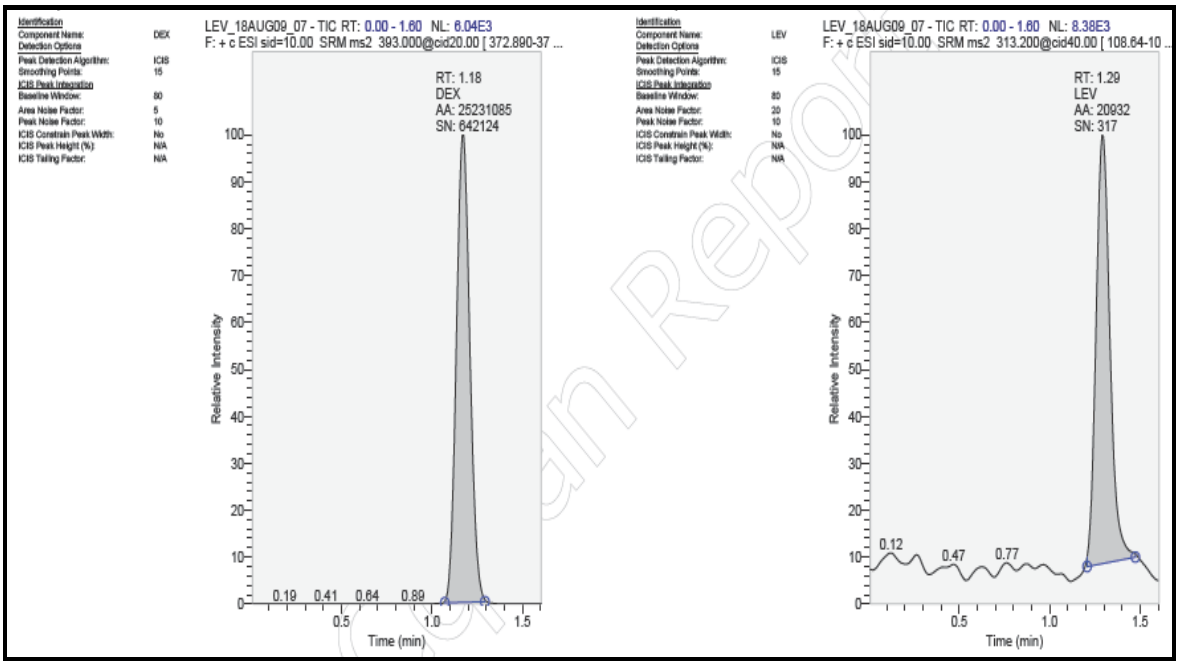

Figure No.24 (b). Representative chromatogram of Levonorgestrel LLOQ (0.100ng/mL)

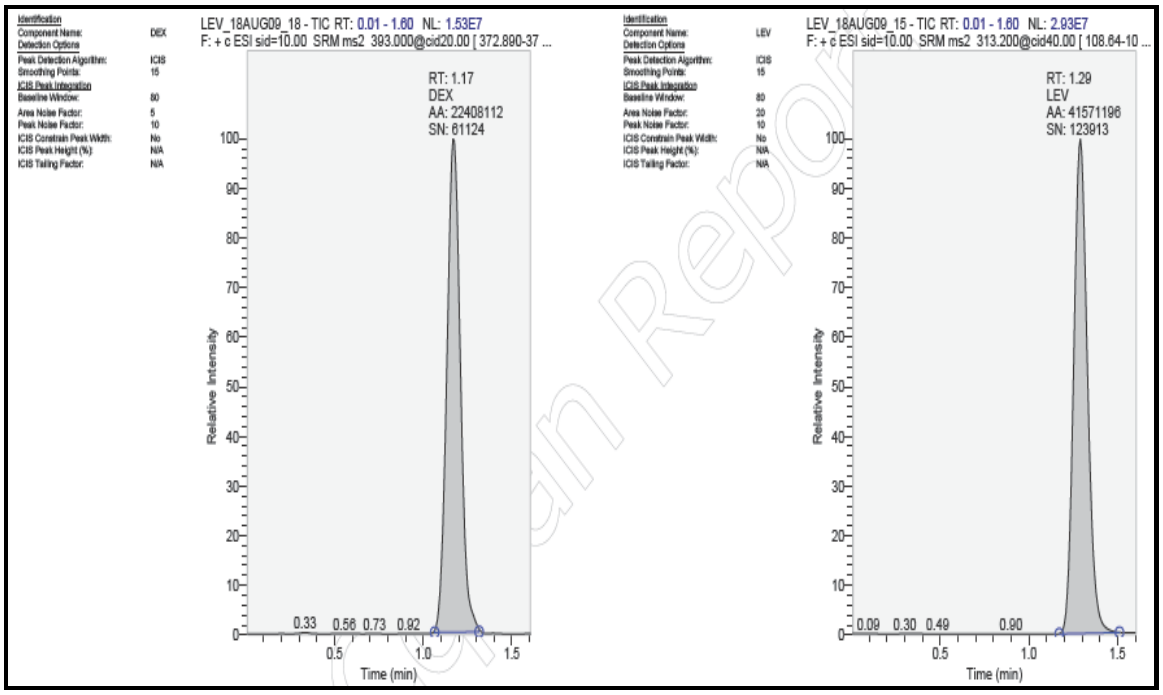

Figure No. 24(c). Representative chromatogram of Levonorgestrel ULOQ (200.000ng/mL)

6.6.2 System Suitability

System suitability was performed during the beginning of every new sequence by injecting fine injections of aqueous equivalent MQC solution. The %CV for the peak response ratio was found to be ≤ 1.20% and for the retention time was ≤ 0.27.

6.6.3 Specificity/Selectivity

Eight different lots of human K_2EDTA plasma including hemolysed and lipemic plasma were analyzed to determine the extent to which endogenous components in plasma which contributes to chromatographic interference with the analytes or internal standard. No significant interference from the blank plasma was observed at the retention time of analytes and internal standard. The results are presented in the *table no.26.*

6.6.4 Carry Over Check

Carryover check of the sample was carried out by injecting the highest concentration of the calibration curve (ULOQ) and internal standard followed by the reconstitute solution and

extracted blank plasma. No significant carryover was observed in reconstitute solution and extracted blank plasma.

6.6.5 Sensitivity

The lower limit of quantification was 0.100ng/mL with a percentage coefficient of variation 5.36% and mean percentage nominal concentration 84.42% Signal to noise ratio was ≥ 317.

6.6.6 Calibration curve

Calibration curves were found to be over a calibration range of 0.100 to 200.000 ng/mL. The calibration model was determined by testing the algorithms linear/quadratic, 1/x weighted linear/quadratic, $1/x^2$ weighted linear/quadratic. The %CV of the slope across the batches was 6.77. The coefficient of determination(r^2) was ≥ 0.9963.The calibration model of $1/x^2$ weighted linear regression gave a good fit and the model was reproducible that minimizes the bias of the back-calculated values. The results were presented in the *table no.27.* A typical calibration curve is presented in *figure no.25.*

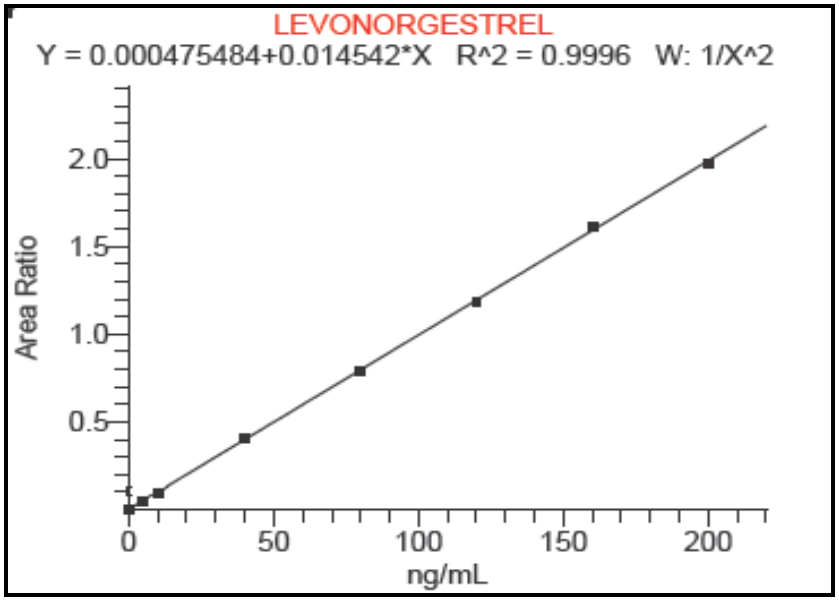

Figure No. 25. Representative calibration curve of Levonorgestrel

Table No. 26. Specificity/Selectivity of human blank plasma of Levonorgestrel and Dexamethasone

SAMPLE NAME	Analyte Area	ISTD Area	SAMPLE NAME	Analyte Area	ISTD Area
EXT-LLOQ-LOT#K2E-01-01	17452	23514212	EXT-LLOQ-LOT#K2E-005	18532	23568954
EXT-LLOQ-LOT#K2E-01-02	24521	22231542	EXT-LLOQ-LOT#K2E-005	17452	25632548
EXT-LLOQ-LOT#K2E-01-03	19352	22236584	EXT-LLOQ-LOT#K2E-005	18235	24325658
Mean	20442	22660779	**Mean**	18073	24509053
BLANK SAMPLE-LOT#K2E-01	3658	100023	BLANK PLASMA LOT#K2E-005	2058	205952
% of Area	17.90	**0.44**	**% of area**	**11.39**	**0.84**
EXT-LLOQ-LOT#K2E-02-01	16235	22365482	EXT-LLOQ-LOT#K2E-006	18236	25326541
EXT-LLOQ-LOT#K2E-02-02	17452	25326475	EXT-LLOQ-LOT#K2E-006	19532	22365248
EXT-LLOQ-LOT#K2E-02-03	16852	26325478	EXT-LLOQ-LOT#K2E-006	16325	23145621
Mean	16846	24672478	**Mean**	18031	23612470
BLANK SAMPLE-LOT#K2E-02	1021	125489	BLANK SAMPLE-LOT#K2E-006	1110	163254
% of Area	**6.06**	**0.51**	**% of area**	**6.16**	**0.69**
EXT-LLOQ-LOT#K2E-03-01	16234	23654782	EXT-LLOQ-LOT#K2E-L-001	18254	25326521
EXT-LLOQ-LOT#K2E-03-02	17523	21453265	EXT-LLOQ-LOT#K2E-L-001	14253	23215464
EXT-LLOQ-LOT#K2E-03-03	17265	24325614	EXT-LLOQ-LOT#K2E-L-001	15231	22362541
Mean	17007	23144554	**Mean**	15913	23634842
BLANK SAMPLE-LOT#K2E-03	1325	1256321	BLANK SAMPLE-LOT#K2E-L-001	1023	1256325
% of Area	**7.79**	**5.43**	**% of area**	**6.43**	**5.32**
EXT-LLOQ-LOT#K2E-04-01	18523	22658418	EXT-LLOQ-LOT#K2EH-001	16325	23625418
EXT-LLOQ-LOT#K2E-04-02	17523	23654125	EXT-LLOQ-LOT#K2EH-001	15236	23625419
EXT-LLOQ-LOT#K2E-04-03	18523	22365241	EXT-LLOQ-LOT#K2EH-001	18235	25326521
Mean	18190	22892594.67	**Mean**	16599	24192453
BLANK SAMPLE-LOT#L-004	985	214574	BLANK SAMPLE-LOT#K2EH-001	1524	1212625
% of area	**5.42**	**0.94**	**% of area**	**9.18**	**5.01**

Table No.27. Back calculated concentrations of Levonorgestrel calibration standards

Linearity	Concentration(ng/mL)								
	STD-1	**STD-2**	**STD-3**	**STD-4**	**STD-5**	**STD-6**	**STD-7**	**STD-8**	**STD-9**
	0.100	0.200	1.000	10.000	40.000	80.000	120.000	160.000	200.000
Mean	0.102	0.200	1.001	10.008	41.200	83.200	119.600	163.600	195.200
SD	0.004	0.007	0.002	0.056	1.924	3.033	5.030	5.941	5.404
%CV	3.92	3.50	0.20	0.56	4.67	3.65	4.21	3.63	2.77
Mean % nominal Conc.	102.00	100.00	100.10	100.08	103.00	104.00	99.67	102.25	97.60

6.6.7 Precision and Accuracy (P&A)

Within-batch or intra-batch accuracy and precision evaluation were assessed by analyzing one calibration curve and 4 sets of QC samples (6 replicates each of the LLOQC, LQC MQC and HQC) in five different batches. Mean percentage nominal concentration of at LLOQC for Batch-01, Batch-02, Batch-03, Batch-04 and Batch-05 were 99.05%, 99.56%, 98.99%, 97.67and 101.00% respectively. Percentage coefficient of variation at LLOQC for Batch-01, Batch-02, Batch-03, Batch-04 and Batch-05 were 4.04%, 6.06%, 5.10%, 7.22% and 4.95% respectively. Mean percentage nominal concentration of Levonorgestrel at LQC, MQC and HQC for Batch-01, Batch-02, Batch-03, Batch-04 and Batch-05 were 104.84 to 103.40%, 99.89 to 101.17%, 98.83 to 102.17%,100.33 to 102.57% and 99.33 to 105.10% respectively. Percentage coefficient variation at LQC, MQC and HQC for Batch-01, Batch-02, Batch-03, Batch-04 and Batch-05 were ≤ 4.80%, ≤ 6.04%, ≤6.04%, ≤5.35% and ≤3.96% respectively. Between batch or inter-batch results are presented in the *table No.28.*

Table No. 28. Between-batch or inter-batch accuracy and precision of Levonorgestrel

QC levels(Conc.) (n=24)	**LLOQC (0.100 ng/mL)**	**LQC (0.300 ng/mL)**	**MQC (100.000 ng/mL)**	**HQC (180.000 ng/mL)**
Mean Calc. Conc.	0.099	0.301	102.648	179.879
SD	0.001	0.008	4.796	4.224
%CV	1.01	2.66	4.67	2.35
Mean % Nominal Conc.	99.04	100.43	102.63	99.93

6.6.8 Recovery

The percentage recovery of LEV and DEX was determined by comparing the mean peak area of extracted LQC, MQC and HQC samples with freshly prepared post spiked (unextracted) LQC, MQC and HQC samples respectively. Mean % recovery at LQC,MQC and HQC was found to be 82.97%, 89.58% and 84.78% respectively. Mean % recovery of LEV across QC level was 85.78 % and variability across QC levels was 3.98%. The mean percentage recovery for DEX was 79.89%.

6.6.9 Stability

6.6.9.1 Stock solution stability

LEV and DEX main stock and LEV spiking stock solution at MQC level were prepared and aliquots of stocks were stored at 2-8°C (stability sample). After 15 days LEV MQC spiking stock and DEX working stock solution was prepared from the main stock (stability samples) and all the stability samples were analyzed with the freshly prepared aqueous equivalent MQC spiking stock and working solution of ISTD. LEV, DEX main stock solution and LEV spiking stock at 2-8ºC was found to be stable for 15 days.

To assess the short term stability of main stock solution of LEV, DEX, spiking stock of LEV and DEX internal standard working solution were kept at the room temperature (stability samples). After 12 hrs LEV MQC spiking stock and DEX working stock solution was prepared from stability samples and all the stability samples were analyzed with freshly prepared LEV MQC spiking stock solution and DEX working stock solution. The area ware compared and LEV, DEX main stock solution and LEV spiking stock and DEX internal standard working solution found to be stable for 12 hrs at room temperature. The mean % change between the fresh and stability samples was found to be within ± 10% indicating the analyte was stable at room temperature and at2-8°C.The results were presented in the *table no.29.*

Table No.29 Stock solution stability of Levonorgestrel

Stability	Mean area		Mean % Change
	comparison sample	Stability sample	
Main stock solution stability for 15 days at 2-8°C	32569856	31805231	-2.35
Spiking stock solution stability for 15days at 2-8°C	32569856	30856478	-5.26
Main stock solution stability for 12 hrs at room temp.	32569856	31909742	-2.03
Spiking stock solution stability for 12 hrs at room temp.	32569856	31512848	-3.25

6.6.9.2 Stability of drug in plasma

The procedure and acceptance criteria for all stability parameters were same as mentioned in the section 4.6.9.2. Bench top (BT) stability for 8 hrs, in-injector stability for 38 hrs at 5°C, Freeze thaw (FT) stability for 4 cycles, dry extract (DE) stability for 4 hrs at room temperature, wet extract (WE) stability for 6 hrs at room temperature, process stability of the samples has for 4 hrs at room temperature and long term (LT) stability for 68 days at -70±5°C has been performed. The results of all stability experiments were found to be within the acceptance criteria and Ethinyl estradiol was found to be stable at various conditions. The results were presented in *table no. 30.*

Table No.30.Plasma stability of Levonorgestrel

Analytes	BT-Stability	In-injector stability for	FT Stability	DE stability	WE stability	Process stability	LT stability
Mean % change							
LQC	2.18	0.32	-3.26	-6.26	-5.26	-8.22	-2.05
HQC	-4.20	-4.03	-4.50	-4.50	-7.50	-4.05	-6.08

6.6.10 Dilution integrity:

Dilution integrity experiment was carried out at six replicates of two times (½ dilution) and of four times diluted (¼ dilution) 2 X ULOQ samples. The samples were prepared and concentrations were calculated including the dilution factor against the calibration curve. The mean percentages nominal concentration of LEV was found to be 108.76 and 102.85% for ¼ and ½ dilution. Percentage coefficient of variation was ≤ 8.97. This indicated the integrity of results at ½ and ¼ dilution.

6.6.11 Matrix effect

Matrix effect was carried out at LQC and HQC levels in six different lots of K_2EDTA plasma including hemolysed and lipemic plasma. Drug free K_2EDTA plasma was processed and the extracted matrix was post spiked with LQC and HQC spiking stock solutions and internal standard. The aqueous equivalent LQC and HQC was also injected and analyzed. Matrix factor (ion suppression/ enhancement) was determined by comparing the area response of post-spiked LQC, HQC with aqueous equivalent LQC and HQC. No significant matrix effect was observed. The % CV across different lots of internal standard normalized matrix factor at

LQC and HQC is 2.62 % and 5.35% respectively.

6.6.12 Hemolysis and lipemic effect

The precision and accuracy experiment was performed using K_2EDTA plasma for calibration standards and hemolysed and lipemic plasma for QC samples (Six replicates each of LQC and HQC).Mean Percentage nominal concentration of LEV for haemolised samples at LQC and HQC were 98.68 % and 104.69% respectively. Mean Percentage nominal concentration of LEV at LQC and HQC for lipemic samples was 104.24% and 107.69 % respectively Percentage coefficient of variation of LEV was ≤ 6.11% for hemolised samples and ≤2.36%.for lipemic samples. This showed that hemolysed and lipemic samples have no impact on the quantification of the analyte.

6.6.13 Ruggedness

Ruggedness of the method was determined by analyzing the two batches of P&A samples processed by two different analysts; one P&A analyzed using a different lot of the column and another P&A prepared from different lots of solvents. The mean percentage nominal concentration and the coefficient of variation across these batches were calculated. Mean percentage nominal concentration at LLOQC level was found to be 96.24% and at LQC, MQC and HQC were found to be 100.69 to 105.26%. Coefficient of variation at LLOQC level was 2.56% and at LQC, MQC &HQC was < 3.29%. All the values were found be within the acceptance criteria, indicating the ruggedness of the method with the external variables.

6.6.14 Production batch run

A production batch comprising of 160 samples, which includes linearity and randomly placed QC's were analyzed. This run was planned in order to simulate the number of samples that could be acquired in a production batch, during subject sample analysis. Mean % nominal concentration was found to be 94.12 to 106.12% and the percentage coefficient of variation was found to be ≤0.14%. The values were found to be within the acceptance criteria indicating the method was stable enough to analyze 160 samples in a batch.

6.6.15 Reinjection Reproducibility

After the completion of analysis of any of the P&A batch, the calibration standards, LQC and HQC samples were retained in auto sampler. After 12 hrs these samples were re-injected. The

results were compared with. The percentage differences between initial analysis and results of reinjected QCs samples were calculated. The % difference between the analysis at LQC and HQC was found to 2.62% and HQC 5.35% respectively. These showed analytical batches can be re-injected in case of instrument failure or any reasons which interrupts run.

6.7 Conclusion

Levonorgestrel is a synthetic derivative of the hormone progesterone. Levonorgestrel causes side effects like low stomach/abdominal pain, breast tenderness, vomiting, diarrhea and menstrual changes. Treatment with low dose Levonorgestrel has emerged as effective, convenient and safe method for emergency contraception. This needs estimation of the Levonorgestrel at very low concentrations in plasma. The reported methods are not sensitive enough to quantify Levonorgestrel at very low concentrations. Clinical application of the bioanalytical methods demands selectivity, sensitivity and rapidness. Therefore a rapid, sensitive and highly selective method for the determination of Levonorgestrel in K_2EDTA plasma was developed, using LC-MS/MS.

The method was more selective than previously described HPLC and RIA methods. Scanning the analytes with positive ionization mode showed high relative abundance and stable signals when compared to the negative ionization mode. The most abundant and stable product ions were selected for selective reaction monitoring (SRM) of the analytes. The mass transitions of 313→109 and 393→293 were selected for LEV and DEX respectively. The MS/MS parameters were optimized by direct infusion of the analytes through the mobile phase in to mass spectrophotometer source and adjusting the spray needle position to achieve optimal response.

A Hypersil gold, C18 (50×4.6mm, i.d., 4μ) column with mobile phase consisting of Methanol: 0.1% formic acid in water (87:13% v/v) gave good chromatography with the run time of 1.60 minutes for high throughput analysis. The method exhibited high specificity of MS/MS detection; no interfering peaks were found on chromatographing blank plasma extracts from eight different sources. The matrix effect was tested in all eight sources of human plasma including hemolysed and lipemic samples. No significant matrix effect was observed. A weighting factor of $1/x^2$ was used to construct the calibration curves. The coefficient of determination (r^2) was found to be ≥0.9963. The method was found to be linear

over a concentration range of 0.100-200.000ng/mL. Calibration range was selected, based on the C_{max} of different dosage strengths of Levonorgestrel. The lowest concentration quantified was 0.100 ng/ml with 0.300mL plasma having acceptable accuracy and precision. The intra-day precision ranged from 4.04% to 7.22%, while inter-day precision ranged from 3.96 to 6.04%. The intra-day accuracies ranged from 97.67 to 101.00%, while the inter-day accuracies ranged from 99.04 to 102.63%.

The method is sensitive enough to quantify 0.100ng/mL using very low volume of plasma. The chromatographic run time was 1.60 mins allowing large number of samples analysis per day. The method was successfully applied to chronopahrmacoketic study. Thus the method was proven to be sensitive, selective, rapid and rugged and useful for clinical pharmacokinetic studies.

Chapter-7

BIOANALYTICAL METHOD FOR DETERMINATION OF MIFEPRISTONE IN HUMAN PLASMA

Mifepristone is a synthetic 19-norsteroid that exhibits great affinity for the progesterone and glucocorticoid receptors. Mifepristone is a progesterone receptor antagonist used as an abortifacient and also as an anti-glucocorticoid used to reduce glucocorticoid receptor activation as a potential therapy in metabolic syndrome and depression at low doses. This calls for the measurement needs to measure low concentration of the analyte in plasma. A low dose mifepristone regimen is effective and side effects will be reduced [121]. The reported methods are not sensitive enough or require more analysis run time [101]. Many clinical studies on pregnancy termination and emergency contraception have focused on the decrease of the dose of mifepristone from 200–600 mg to 2–100 mg[122]. Thus, developing a highly sensitive and validated method to determine lower levels of mifepristone in plasma is required. Therefore the aim of this work was to develop an assay by Liquid chromatography–tandem mass spectrometry (LC–MS/MS) to enhance the specificity and sensitivity ofor the analyte and to allow analysis of smaller volumes of plasma with lower concentrations of the analyte.

7.1 Reference/Working standards

Mifepristone working standard was procured from Cipla Limited, Mumbai, India while Fluticasone propionate working standard was obtained from the Sun Pharma, Baroda, India as gift sample.

7.2 Reagents and chemicals

Methanol, acetonitrile, ammonium formate, ammonium acetate, water, n-Hexane, ethyl-acetate, tertiary-butyl methyl ether and dichloromethane were used in the method. The grade and manufacturers were same as mentioned in section- 4.2.

7.3 Instruments

The instruments used for method development and validation were Auto sampler, Analytical balance, Analytical Columns((Genesis,C-18, (100×4.6mm, 4µ), ACE, C-18, (35×4.6mm, 3µ), Kromacil,C-18, (50×4.6mm, 4µ) and Hypersil gold,C-18, (50×4.6mm,4µ)),Column oven, Centrifuge, Deep Freezer, Hot air oven, Micro balance, Micropipette, MS Detector, Mulitipulse Vortexer, Nitrogen Evaporator, Solid phase extraction unit, Solvent delivery module, Ultrasonic bath, Vortexer and Water purification system. The Make/Model and manufacturer are same as that of section 4.3.

7.4 Blank plasma

Harvested K_2 EDTA blank plasma for method development and validation was obtained Navajeevan blood bank, Hyderabad, India and Blood Bank, Kasturba Hospital, Manipal, India.

7.5 Method Development

7.5.1 Scanning and Optimization of MS/MS detection parameters

Method development involves scanning of the analyte and internal standard solutions to find the parents ions and its respective fragment ions; for this purpose 100ng/mL analyte and internal standard solution in methanol was prepared separately. Solution was injected into mass spectrophotometer source using the syringe pump. Once the parent ion was obtained it was further scanned for product ions using MS/MS mode. The fragment ion having high intensity was selected for SRM. Once these chromatographic conditions were set, the analyte was tuned manually in SRM mode with the mobile phase by using "T" which connects LC pump and syringe pump to the detector in order to optimize tube lens offset voltage, collision gas pressure, collision energy, sheath gas pressure, auxiliary gas pressure and capillary temperature to achieve maximum response.

The mass spectra of MIF and FLU were recorded from 50 to 600m/z and mass scan analysis is presented in the *figure no.26 (a) and 18(b)*. Although mifepristone is hydrophobic in nature it is ionized under electro spray conditions yielding protonated $[M+H^+]$ molecular ions at m/z at 430 and product ion at 236 m/z . Molecular ions m/z at 501 and product ion at 293m/z for Fluticasone propionate was obtained. The detector parameters were optimised to obtain higher response and tabulated in the *table no. 31*. Both Q1 and Q3 were operated under unit mass resolution.

Table No.31. Ion source parameters for Mifepristone and Fluticasone propionate.

Sr. No.	Ion source parameters	
1	Interface	ESI
2	Ionization mode	Positive
3	Spray voltage(V)	4500
4	Sheath gas pressure(arb)	40
5	Auxillary gas pressure(arb)	20
6	Capillary temperature(C)	350
7	Collision gas pressure(mTorr)	1.5
8	Chrom.filter	10
9	Skimmer offset	10
10	Collision Evergy(eV)	28 for MIF and25 for FLU
11	Tube lens offset(V)	100 for MIF and FLU

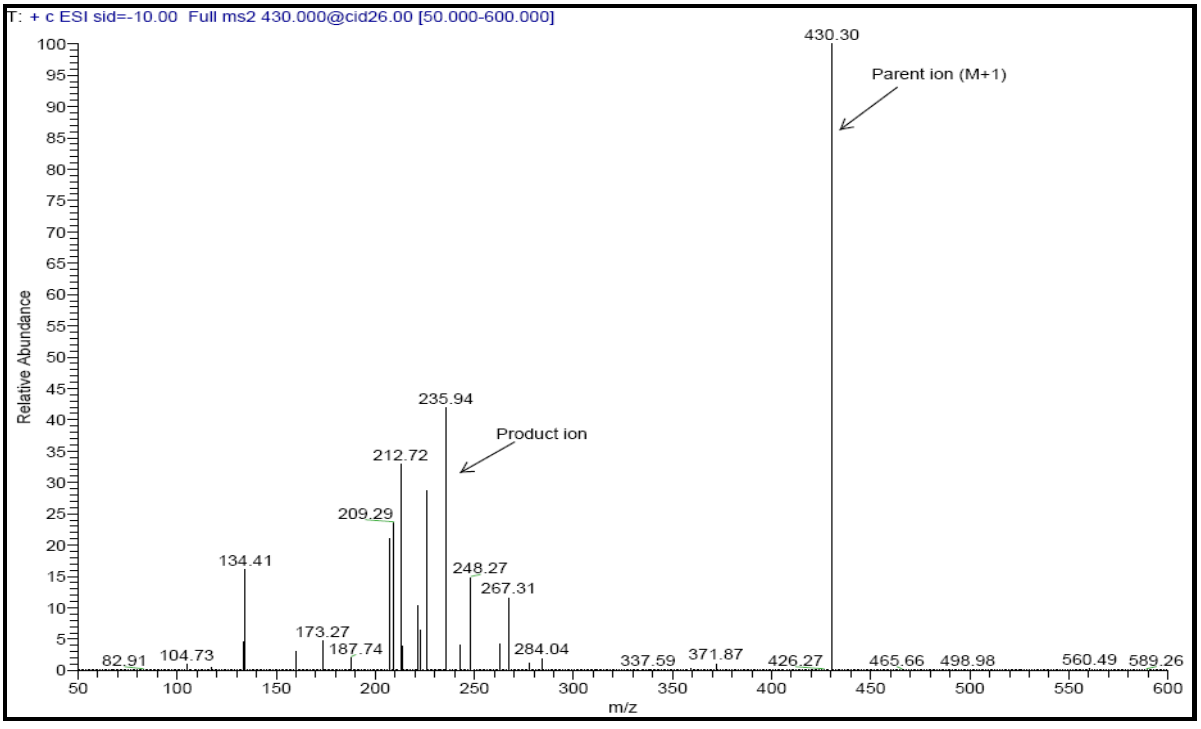

Figure No: 26(a). Parent and product scan analysis for Mifepristone

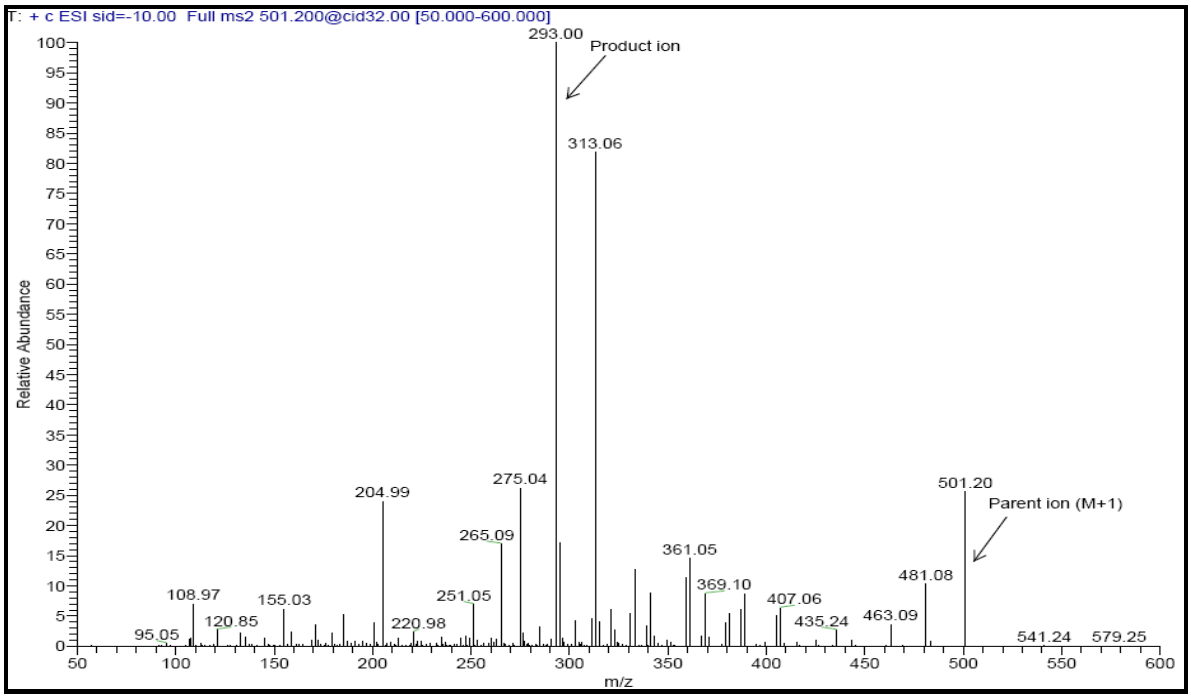

Figure No: 26(b). Parent and product scan analysis for Fluticasone propionate

7.5.2 Preparation of main stock solutions of mifepristone and Fluticasone propionate

Mifepristone main stock 2.500mg/mL solution was prepared by weighing the working standard equivalent to 25.000 mg into 10.000mL volumetric flask, dissolved with 5.000mL of methanol and diluted up to the mark with methanol.

Fluticasone propionate main stock 1.000mg/mL solution was prepared by weighing the reference standard equivalent to 10.000 mg into 10.000mL volumetric flask, dissolved with 5.000mL of methanol and diluted up to the mark with methanol.

7.5.3 Preparation of intermediate stock solution (125000.00ng/mL) and internal standard working solution(0.750 µg/mL)

0.125 mL of the mifepristone main stock solution was transferred into a 25.000mL volumetric flask and volume was made up to 25.000mL with methanol: water (50:50%v/v) and vortexed to mix well.

0.750 mL of the Fluticasone propionate main stock solution was transferred in to 10.000mL volumetric flask and volume was made up to 10.000mL with methanol: water (50:50%v/v).

7.5.4 Preparation of calibration standards spiking stock

Calibration standards were prepared as per the table mentioned below

Calibration standard	Volume of intermediate stock taken (mL)	Volume made up to (mL)	Spiking stock concentration (ng/mL)	Plasma conc.(ng/mL)
STD-1	0.016	100.000	20.000	1.000
STD-2	0.032	100.000	40.000	2.000
STD-3	0.200	5.000	5000.00	250.000
STD-4	0.400	5.000	10000.000	500.000
STD-5	0.800	5.000	20000.000	1000.000
STD-6	1.600	5.000	40000.000	2000.000
STD-7	2.400	5.000	60000.000	3000.000
STD-8	3.200	5.000	80000.000	4000.000
STD-9	4.000	5.000	100000.000	5000.000

7.5.5 Quality control samples spiking stock preparation

Quality control samples were prepared as per the table mentioned below

Quality Control	Volume of intermediate stock taken (mL)	Volume made up to (mL)	Spiking stock conc. (ng/mL)	Plasma conc. (ng/mL)
LLOQ	0.016	100.000	20.000	1.000
LQC	0.048	5.000	60.000	3.000
MQC	2.000	5.000	50000.000	2500.000
HQC	3.600	5.000	90000.000	4500.000

7.5.6 Preparation of solutions and buffers

Weighed amount of the salt was transferred in to a glass beaker and 500.000mL of milli-Q/HPLC water was added and sonicated to mix well. The solution was then filtered through 0.22µm membrane filter. Different strengths of the buffer were prepared by weighing the salts as per the table shown in section 4.5.5.1, and 0.1% formic acid in water was prepared as per the procedure mentioned in section 4.5.5.2.

7.5.7 Preparation of buffer solutions

Weighed amount of the salt was transferred in to a glass beaker and 500.000mL of milli-Q/HPLC water was added and sonicated to mix well. The solution was then filtered through 0.22µm membrane filter. Different strengths of the buffer were prepared by weighing the salts as shown below.

7.5.8 Optimizing chromatographic conditions

7.5.8.1 Effect of pH, stationary phases, solvent strength and flow rate.

To achieve good peak shape and response of mifepristone various mobile phases like Methanol: water, ACN: water, Methanol; ammonium acetate (2mM, 5mM, 10mM), Methanol: Ammonium formate (5mM, 10mM), ACN: 0.1% formic acid in water, methanol: formic acid in water at different mobile phase compositions (90:10%v/v, 80:20%v/v,70:30%v/v) were tried. Different C18 columns like Genesis (100×4.6mm, i.d., 4µ), ACE (35×4.6mm, i.d., 3µ), Kromacil (50×4.6mm,i.d., 4µ particle size) and Hypersil gold (50×4.6mm, i.d.,4µ) columns were investigated. Genesis C18 (100×4.6mm, i.d., 4µ) column with methanol: 0.1% formic acid in water (80:20%) showed symmetrical peak shape and good response, but the run time was more than 4.00mins. Chromatography with ACE C18 (35×4.6mm, i.d., 3µ) column at the mobile phase composition of methanol: 10mM ammonium acetate solution (90:10%v/v) exhibited good chromatographic conditions with less run time. LC flow rates were tested and a flow rate of 0.400mL/ min was given optimum response at the LLOQ level. Column oven temperature was set at 40°C and auto sampler tray temperature was set at 5°C. Rinsing solution was optimized to 0.1% formic acid in Methanol in order to remove the auto sampler carryover. The flush and wash volume were at 2000 µL/Sec. Injection volume was 10.00µL and overall chromatographic run time was 1.60 min.

7.5.8.2 Selection of internal standard

The internal standard was selected on the basis of chemical structure, polarity and solubility characteristics. Based on the physicochemical properties Fluticasone propionate was selected and chromatographed. The response of the Fluticasone propionate was found to be reproducible with the optimized mobile phase conditions.

7.5.8.3 Final chromatographic conditions

Chromatographic mode	: Reversed Phase
Isocratic/gradient mode	: Isocratic
Internal Standard	: Dexamethasone
Rinsing solution	: 0.1% formic acid in methanol
Injection volume	: 10.00 µL

Column	: ACE, C18 (35×4.6mm i.d., 3µ)
Mobile phase	: Methanol and 10mM ammonium acetate solution (90:10% v/v)
Column oven temperature	: 40°C
Auto sampler tray temperature	: 5°C
Flush volume	: 2000 µL/Sec
Wash volume	: 2000 µL/Sec
Flow rate	: 0.400mL/min.
Run time	: 1.60minutes
Retention time of mifepristone	: 1.17min
Retention time of fluticasone propionate	: 1.00min

7.5.8.4 Selection of extraction technique

Extraction procedure was initiated with simple protein precipitation technique using precipitating agents like methanol and acetonitrile. Matrix effect was observed with protein precipitation and recovery was less. Various trials were taken by liquid-liquid extraction technique using n- hexane, TBME, DCM and ethyl acetate. To increase the extraction efficiency, various compositions of TBME: DCM, n- hexane: TBME were tried. Recovery with TBME: DCM (60:40%v/v) was found to be good but matrix effect was also observed. However matrix effect was not observed with genesis C18 (100×4.6mm,i.d., 4µ) column but it required long run time. Solid phase extraction was tried by extracting the samples with oasis HLB 1cc cartridges using water, ammonium acetate and 0.1% formic acid buffer in different trials. After buffering the samples were loaded on previously conditioned (with 1.00mL methanol and 2.00mL of water) oasis HLB 1cc cartridges and washed with 2.00mL of water and 2.00mL of 5% methanol in water and eluted with 1.00mL of methanol. The eluate was evaporated under ae stream of nitrogen at 40°C. The residue was reconstituted with 0.300mL of Methanol:10mM ammonium acetate (90:10%v/v). mixture 10.00µL of the resulting solution was injected in to LC-MS/MS.

Final extraction Procedure: 0.015mL of analyte and 0.275 mL plasma samples were transferred in to a 2.00 mL micro centrifuge tube and vortexed to mix. 15 µL of internal

standard working solution was added and vortexed to mix. 0.300mL of the 0.1% v/v formic acid in water was added and vortexed to mix. This solution was loaded onto previously conditioned (with 1.0mL of methanol and 2.00mL of water) HLB 1cc cartridges. The cartridges were washed with 2.00mL of water followed by 2.00mL of 5% v/v methanol in water and eluted with 1.0mL of methanol. The eluate was evaporated to dryness under a stream of nitrogen at 40°C. Residue was reconstituted with 0.300mL of mobile phase and vortexed for 1.0 minute. 10.0µL of this solution was injected into the LC–MS/MS system.

7.6 Method Validation

A high performance liquid chromatographic method with mass detection for the determination of Mifepristone in human K_2EDTA plasma was developed as per the Guidance for Industry entitled 'Bioanalytical Method Validation' of the United States Food and Drug Administration, Center for Drug Evaluation and Research (CDER) May-2001[1].Mifepristone and Fluticasone propionate (Internal Standard) were extracted from an aliquot of human plasma using solid phase extraction technique and injected in to a liquid chromatograph equipped with a tandem mass spectrometry detector. Quantitation was done by peak area ratio method. A weighted ($1/x^2$) linear regression was performed to determine the concentration of analytes. All regressions and figures presented in this validation report were generated by LC-Quan software version 2.5.6. Procedure and acceptance criteria of validation parameters were same as that of chapter-4.

7.6.1 Chromatography

A typical chromatogram obtained from blank sample (Processed blank K_2EDTA human plasma), lower limit of quantification, upper limit of quantification for the analyte and internal standard are represented in f*igure No.27(a),(b and (c)* respectively. The retention was 1.17 and 1.01mins for MIF and FLU respectively. The overall chromatographic run time is 1.60 minutes.

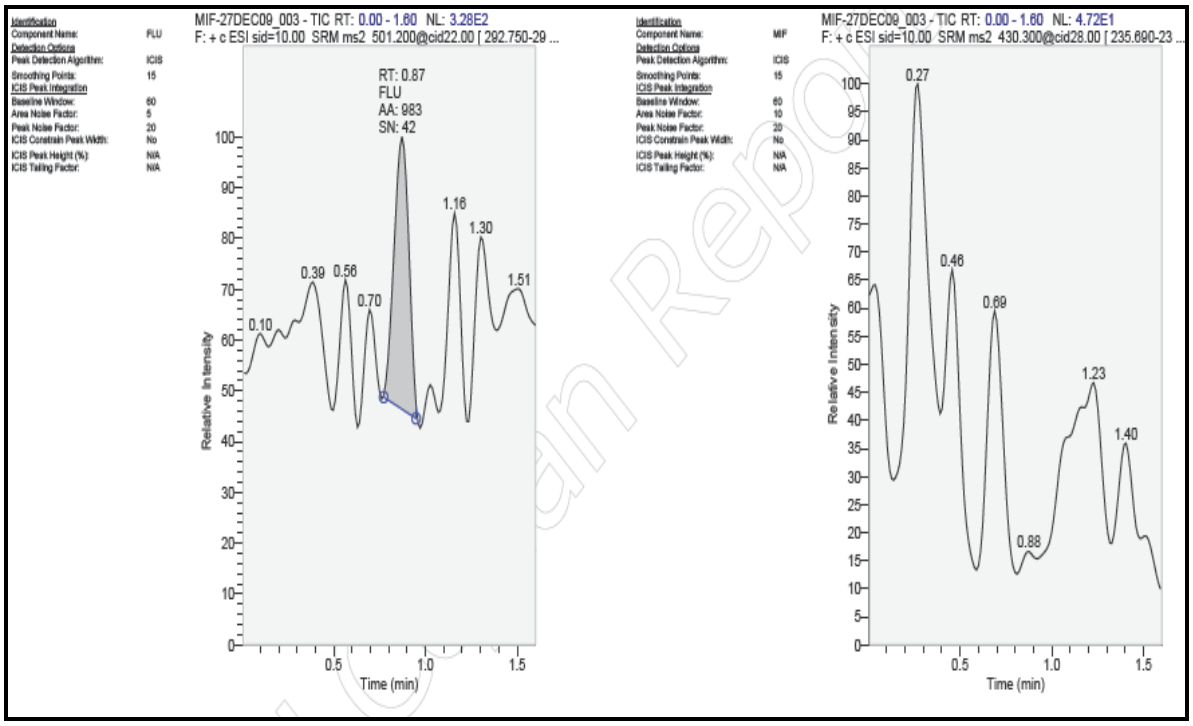

Figure No.27 (a). Representative chromatogram of Mifepristone and Fluticasone propionate blank plasma

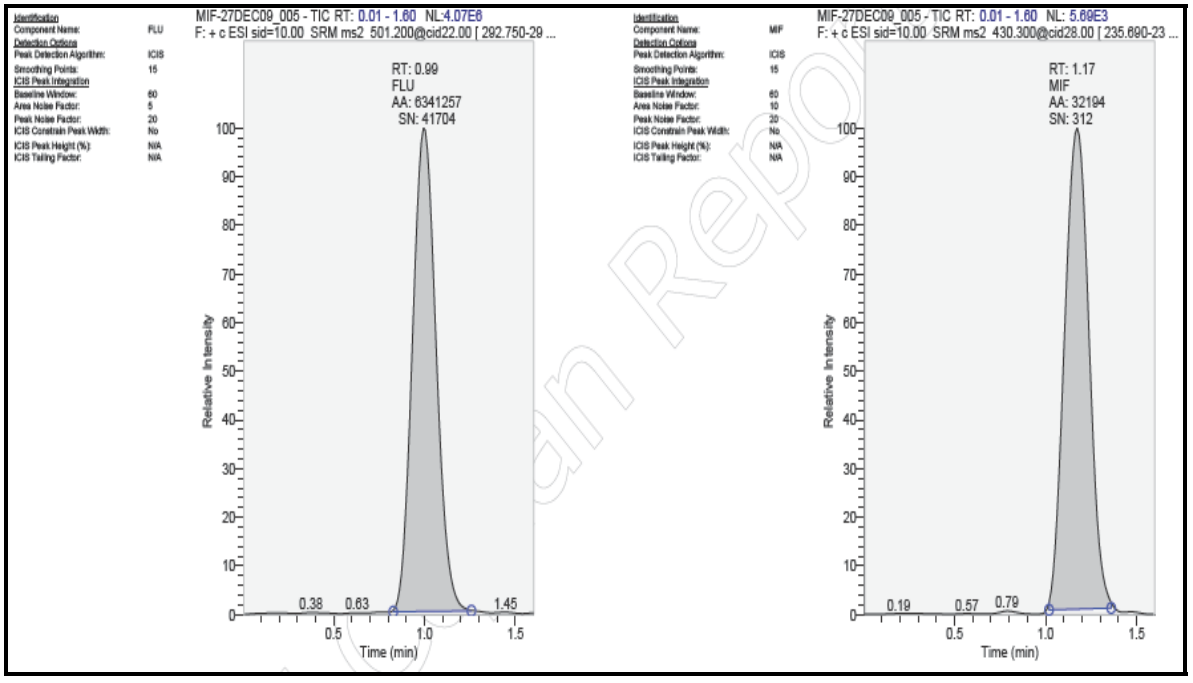

Figure No.27 (b). Representative chromatogram of Mifepristone LLOQ concentration (1.000ng/mL)

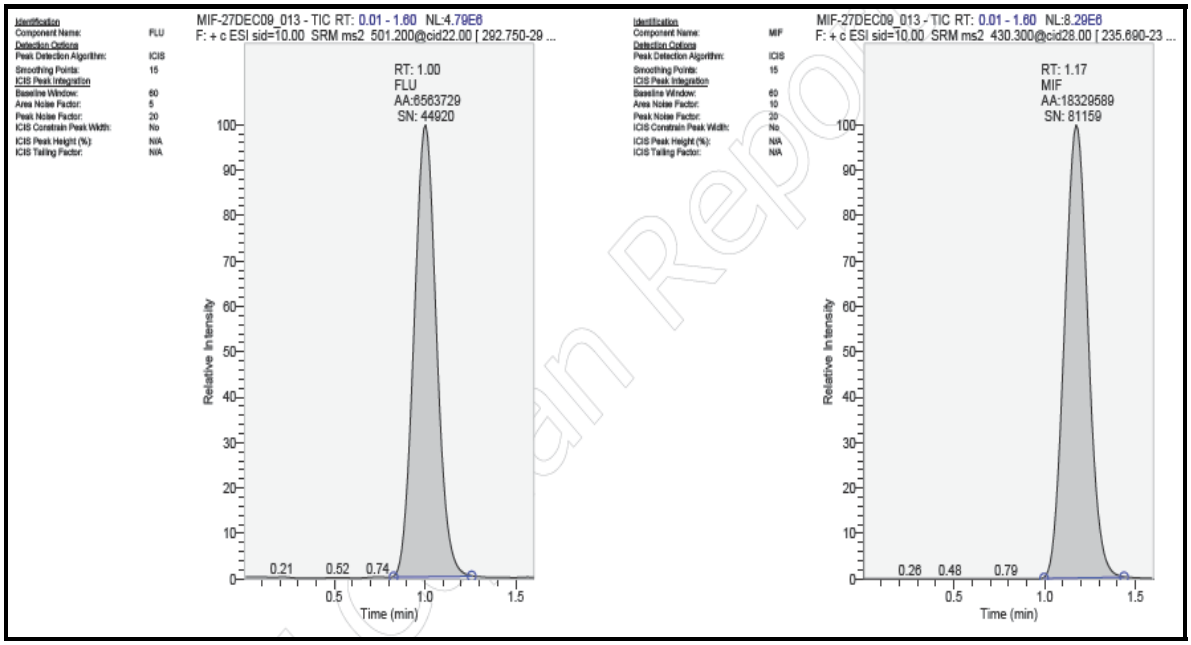

Figure No. 27(c) Representative chromatogram of Mifepristone ULOQ concentration (5000.00ng/mL)

7.6.2 System Suitability

System suitability was performed during the beginning of every new sequence by injecting fine injections of aqueous equivalent MQC solution. The %CV for the peak response ratio was found to be ≤ 2.09% and for the retention time was ≤ 0.24%.

7.6.3 Specificity/Selectivity

Eight different lots of human K_2EDTA plasma including hemolysed and lipemic plasma were analyzed to determine the extent to which endogenous components in plasma which contributes to chromatographic interference with the analytes or internal standard. No significant interference from the blank plasma was observed at the retention time of analytes and internal standard. The results were presented in *table no.32.*

7.6.4 Carry Over Check

Carryover check of the sample was carried out by injecting the highest concentration of the calibration curve (ULOQ) and internal standard followed by the reconstitute solution and extracted blank plasma. No significant carryover was observed in reconstitute solution and extracted blank plasma.

7.6.5 Sensitivity {Lower limit of quantification (LLOQ)}

The lower limit of quantification was 1.00ng/mL with a percentage coefficient of variation 3.00% and mean percentage nominal concentration 85.32% Signal to noise ratio was ≥ 623

7.6.6 Calibration curve

Calibration curves were found to be over a calibration range of 1.000 to 5000.000 ng/mL. The calibration model was determined by testing the algorithms linear/quadratic, 1/x weighted linear/quadratic, 1/x2 weighted linear/quadratic. The %CV of the slope across the batches was 9.22. The coefficient of determination (r^2) was ≥ 0.9975. The calibration model of $1/x^2$ weighted linear regression gave a good fit and the model was reproducible that minimizes the bias of the back-calculated values. Results were presented in *table no.33.* A representative calibration curve is presented in the *figure no.28*

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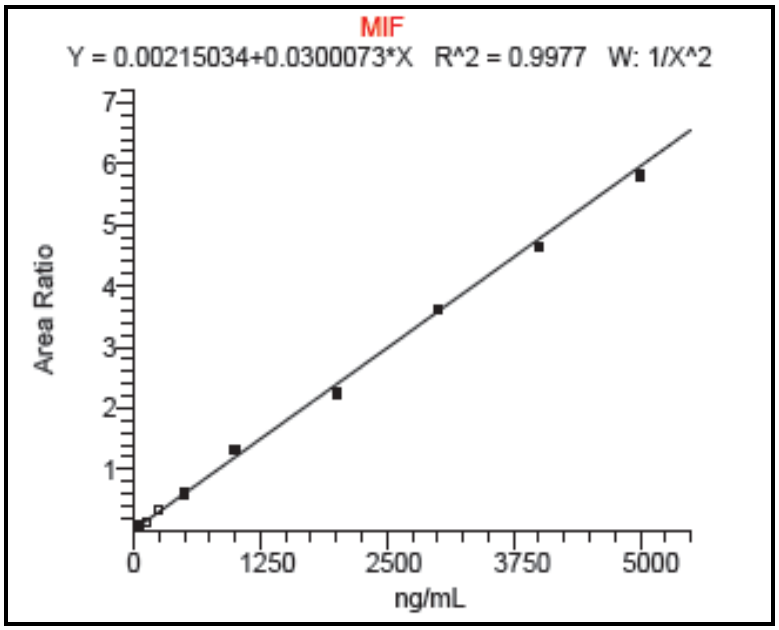

Figure No. 28. Representative calibration curve of Mifepristone

Table No. 32. Specificity/Selectivity of human blank plasma of Mifepristone and Fluticasone propionate

SAMPLE NAME	Analyte Area	ISTD Area	SAMPLE NAME	Analyte Area	ISTD Area
EXT-LLOQ-LOT#K2E-01-01	35261	6532514	EXT-LLOQ-LOT#K2E-005	32512	6825253
EXT-LLOQ-LOT#K2E-01-02	32652	7123654	EXT-LLOQ-LOT#K2E-005	30251	6923514
EXT-LLOQ-LOT#K2E-01-03	32145	6853214	EXT-LLOQ-LOT#K2E-005	29632	6852143
Mean	33353	6836461	**Mean**	30798	6866970
BLANK SAMPLE-LOT#K2E-01	1251	20314	BLANK PLASMA LOT#K2E-005	532	395952
% of Area	**3.75**	**0.30**	**% of area**	**1.73**	**5.77**
EXT-LLOQ-LOT#K2E-02-01	35261	7253159	EXT-LLOQ-LOT#K2E-006	38526	7012354
EXT-LLOQ-LOT#K2E-02-02	34755	7523614	EXT-LLOQ-LOT#K2E-006	35236	6832561
EXT-LLOQ-LOT#K2E-02-03	31254	6932568	EXT-LLOQ-LOT#K2E-006	35236	6532561
Mean	33757	7236447	**Mean**	36333	6792492
BLANK SAMPLE-LOT#K2E-02	256	136254	BLANK SAMPLE-LOT#K2E-006	2452	12325
% of Area	**0.76**	**1.88**	**% of area**	**6.75**	**0.18**
EXT-LLOQ-LOT#K2E-03-01	35624	7214563	EXT-LLOQ-LOT#K2E-L-001	31452	6523145
EXT-LLOQ-LOT#K2E-03-02	31254	6853241	EXT-LLOQ-LOT#K2E-L-001	36521	6632541
EXT-LLOQ-LOT#K2E-03-03	35264	7032541	EXT-LLOQ-LOT#K2E-L-001	31524	6832546
Mean	34047	7033448	**Mean**	33166	6662744
BLANK SAMPLE-LOT#K2E-03	564	256324	BLANK SAMPLE-LOT#K2E-L-001	952	123654
% of Area	**1.66**	**3.64**	**% of area**	**2.87**	**1.86**
EXT-LLOQ-LOT#K2E-04-01	32652	7214563	EXT-LLOQ-LOT#K2EH-001	29562	6235644
EXT-LLOQ-LOT#K2E-04-02	25326	7123652	EXT-LLOQ-LOT#K2EH-001	28524	6532589
EXT-LLOQ-LOT#K2E-04-03	28523	6953251	EXT-LLOQ-LOT#K2EH-001	32105	6325462
Mean	28834	7097155	**Mean**	30064	6364565
BLANK SAMPLE-LOT#L-004	563	253251	BLANK SAMPLE-LOT#K2EH-001	247	125498
% of area	**1.95**	**3.57**	**% of area**	**0.82**	**1.97**

Table No.33. Back calculated concentrations of Mifepristone calibration standards.

Linearity	Concentration(ng/mL)								
	STD-1	**STD-2**	**STD-3**	**STD-4**	**STD-5**	**STD-6**	**STD-7**	**STD-8**	**STD-9**
	1.000	2.000	250.000	500.000	1000.000	2000.000	3000.000	4000.000	5000.000
Mean	0.913	2.117	223.313	468.052	1389.133	2075.789	3081.571	4189.301	4968.278
SD	0.152	0.438	4.164	19.183	12.701	16.339	37.892	40.072	29.724
%CV	3.09	4.33	4.46	9.69	3.26	2.11	3.21	2.52	1.51
Mean % nominal Conc.	98.26	101.17	93.31	99.03	97.28	96.97	98.46	99.33	98.41

7.6.7 Precision and Accuracy (P&A)

Within-batch or intra-batch accuracy and precision evaluation were assessed by analyzing one calibration curve and 4 sets of QC samples (6 replicates each of the LLOQC, LQC MQC and HQC) in four different batches. Mean percentage nominal concentration of at LLOQC for Batch-01, Batch-02, Batch-03 and Batch-04 were 92.11%, 92.57%, 91.09%, and 93.26% respectively. Percentage coefficient of variation at LLOQC for Batch-01, Batch-02, Batch-03, and Batch-04 were 3.33%, 5.35%, 4.89% and 5.81% respectively. Mean percentage nominal concentration of at LQC, MQC and HQC forBatch-01, Batch-02, Batch-03 and Batch-04 were 99.34 to 103.72%, 99.34 to 103.92%, 100.33 to 102.44% and 97.02 to 106.26% respectively. Percentage coefficient variation at LQC, MQC and HQC for Batch-01, Batch-02, Batch-03, and Batch-04 ≤ 7.80%, ≤ 2.14%, ≤4.74% and ≤7.35% respectively. Between batch accuracy and precision results were presented in the *table no.34*.The results have shown the method is precise and accurate.

Table No. 34 Between-batch or inter-batch accuracy and precision of Mifepristone

Sr. No.	LLOQC (1.000 ng/mL)	LQC (3.000 ng/mL)	MQC (2500.000 ng/mL)	HQC (4500.000 ng/mL)
n	24	24	24	24
Mean Calc. Conc.	0.998	2.980	2483.610	4467.053
SD	0.291	0.222	19.197	32.248
%CV	6.24	1.51	1.95	1.64
Mean % Nominal Conc.	93.26	98.33	98.36	98.35

7.6.8 Recovery

The percentage recovery of MIF and FLU was determined by comparing the mean peak area of extracted LQC, MQC and HQC samples with freshly prepared post spiked (unextracted) LQC, MQC and HQC samples respectively. The mean percentage recovery at LQC, MQC and HQC was found to be 88.31%, 81.39% and 84.79% respectively. Mean % recovery of MIF across QC level was 85.78 % and the variability across QC levels was 3.98%. The mean percentage recovery for FLU was 72.23%.

7.6.9 Stability

7.6.9.1 Stock solution stability:

MIF and FLU main stock and MIF spiking stock solution at MQC level were prepared and aliquots of stocks were stored at 2-8°C (stability sample). After 20 days MIF MQC spiking stock and FLUworking stock solution was prepared from the main stock (stability samples) and all the stability samples were analyzed with the freshly prepared aqueous equivalent MQC spiking stock and working solution of internal standard. MIF, FLU main stock solution and MIF spiking stock at 2-8ºC was found to be stable for 20 days.

To assess the short term stability of main stock solution of MIF, FLU, spiking stock of MIF and FLU internal standard working solution were kept at the room temperature (stability samples). After 12 hrs MIF MQC spiking stock and FLU working stock solution was prepared from stability samples and all the stability samples were analyzed with freshly prepared MIF MQC spiking stock solution and FLU working stock solution. The area ware compared and MIF, FLU main stock solution and MIF spiking stock and FLU internal standard working solution found to be stable for 12 hrs at room temperature. The results were presented in the *table no. 35.*

Table No.35. Stock solution stability of Mifepristone

Stability	**Mean area of comparison sample**	**Mean area of Stability sample**	**Mean % Change**
Main stock solution stability for 20 days at 2-8°C	7525364	8076364	7.32
Spiking stock solution stability for 20 days at 2-8°C	7525364	7421326	-1.38
Main stock solution stability for 12 hrs at room temp.	7525364	7567514	0.56
Spiking stock solution stability for 12 hrs at room temp.	7525364	7599254	0.98

7.6.9.2 Stability of drug in plasma

The procedure and acceptance criteria for all stability parameters were same as mentioned in the section 4.6.9.2. Bench top (BT) stability for 35 hrs, in-injector stability for 58 hrs at 5°C, Freeze thaw (FT) stability for 4 cycles, dry extract (DE) stability for 4 hrs at room temperature, wet extract (WE) stability for 6 hrs at room temperature, process stability of the samples has for 4 hrs at room temperature and long term (LT) stability for 90 days at -70±5°C has been performed. The results of all stability experiments were found to be within the acceptance criteria and Mifepristone is stable in plasma at various conditions. The results were presented in table *no. 36.*

Table No.36. Plasma stability of Mifepristone

Analytes	BT-Stability	In-injector stability for	FT Stability	DE stability	WE stability	Process stability	LT stability
Mean % change							
LQC	0.18	1.44	1.44	-1.68	-5.35	-5.36	-8.76
HQC	-3.00	-1.23	1.64	-2.32	-2.85	-3.65	-7.02

7.6.10 Dilution integrity:

Dilution integrity experiment was carried out at six replicates of two times (½ dilution) and of four times diluted (¼ dilution) 2 X ULOQ samples. The samples were prepared and concentrations were calculated including the dilution factor against the calibration curve.

The mean percentages nominal concentration of MIF was found to be 80.12% and 103.67 for ¼ and ½ dilution respectively. Percentage coefficient of variation was ≤ 2.89%. the results were found to be within the acceptance criteria.

7.6.11 Matrix effect:

Matrix effect was carried out at LQC and HQC levels in six different lots of K_2EDTA plasma including hemolysed and lipemic plasma. Drug free K_2EDTA plasma was processed and the extracted matrix was post spiked with LQC and HQC spiking stock solutions and internal standard. The aqueous equivalent LQC and HQC was also injected and analyzed. Matrix factor (ion suppression/ enhancement) was determined by comparing the area response of post spiked LQC, HQC with aqueous equivalent LQC and HQC. No significant matrix effect was

observed. The % CV across different lots of internal standard normalized matrix factor at LQC and HQC is 5.07 % and 4.47% respectively.

7.6.12 Hemolysis and lipemic Effect

The precision and accuracy experiment was performed by using K_2EDTA plasma for calibration standards and hemolysed and lipemic plasma for QC samples preparation (Six replicates each of LQC and HQC).Mean Percentage nominal concentration of MIF at LQC and HQC for hemolysed samples was 95.68 % and 103.69% respectively. Percentage nominal concentration of MIF at LQC and HQC for Lipemic samples was 102.21% and 108.67% respectively Percentage coefficient of variation of MIF was for hemolysed samples was ≤5.81% and for Lipemic samples ≤4.36%. This showed that hemolysed and lipemic samples have no impact on the quantification of the analyte.

7.6.13 Ruggedness

Ruggedness of the method was determined by analyzing the two batches of P&A samples processed by two different analysts; one P&A analyzed using a different lot of the column and another P&A prepared from different lots of solvents. The mean percentage nominal concentration and the coefficient of variation across these batches were calculated. Mean percentage nominal concentration at LLOQC level was found to be 92.05% and at LQC, MQC and HQC were found to be 101.06 to 105.09%. Coefficient of variation at LLOQC level was 3.23% and at LQC, MQC &HQC was ≤ 5.56%. All the values were found be within the acceptance criteria, indicating the ruggedness of the method.

7.6.14 Production batch run (Precision and Accuracy)

A production batch comprising of 160 samples, which includes linearity and randomly placed QC's were analyzed. This run was planned in order to simulate the number of samples that could be acquired in a production batch, during subject sample analysis. Mean % nominal concentration was found to be 98.02 to 101.02% and the percentage coefficient of variation was found to be ≤0.74%. The values were found to be within the acceptance criteria indicating the method was stable enough to analyze 160 samples in a batch.

7.6.15 Reinjection Reproducibility

After the completion of analysis of any of the P&A batch, the calibration standards, LQC and HQC samples were retained in auto sampler. After minimum of 12 hrs these samples were re-

injected. The results were compared with. The percentage differences between initial analysis and results of re-injected QCs samples were calculated. The % difference between the analysis at LQC and HQC was found to -3.282% and HQC -5.80% respectively. This showed the analytical batches can be re-injected in case of instrument failure or any reasons which interrupts run.

7.7 Conclusion

Mifepristone is an oral antiprogestational and antiglucocorticoid agent generally used for short-term therapy. However, treatment of neoplasm or chronic conditions will require long-term administration [169]. Clinical trials have shown that a low dose mifepristone regimen to be effective with minimal side effects [167]. The need for the estimation of the mifepristone at the very low concentration in plasma thus arises. The reported methods are either not sensitive enough to quantify low concentration of the drug in plasma or require long chromatographic run time [126,128]. Therefore a rapid, sensitive and highly selective method for the determination of Mifepristone in K_2EDTA plasma was developed, using HPLC-MS/MS.

Positive ionization mode with selective reaction monitoring was used for the quantification of analytes. The mass transitions of 430→236 and 501→293 were selected for MIF and FLU respectively, based on the relative abundance and stability of the fragment ions. The MS/MS parameters were optimized by direct infusion of the analytes through the mobile phase in to mass spectrophotometer source and by the adjustment of the spray needle position to achieve optimal response. ACE, C18 (535×4.6mm, i.d., 3µ) column with mobile phase consisting of methanol: 10mM ammonium acetate (90:10% v/v)) exhibited good separation of the analyte from the plasma interference with run time of 1.60 minutes. The method exihibited high specificity of MS/MS detection; no interfering peaks were found on chromatographing blank plasma extracts from eight different sources. The matrix effect was tested in all eight sources of human plasma including hemolysed and lipemic samples. No significant matrix effect was observed under these conditions. The calibration curves were prepared by using a weighting factor of $1/x^2$. The coefficient of determination (r^2) was found to be ≥0.9975.The developed method was exhibited a linear response over a concentration range of 1.000-5000.000ng/mL.

The LLOQ determined to be 1.000 ng/mL with 0.300mL plasma. The method is validated and all the validation parameters were found to be satisfactory.

The reported methods are not sensitive enough to measure the low concentration of mifepristone in plasma when the drug administered in low doses. In the present work a LLOQ of 1.000ng/mL was achieved with the low plasma volume of 0.300mL with short analysis run time of 1.60 min. Validation results were shown to be the method is sensitive, selective, rapid and rugged and can be applied for clinical pharmacokinetic studies.

Chapter-8

CHRONOPHARMACOKINETIC STUDIES

8 CHRONOPHARMACOKINETIC STUDY

Chronopharmacology is the study of the variation in the effects of drugs with biological timing and endogenous periodicities. The goal is to improve the understanding of periodic and predictable (e.g. circadian) changes in both desired effects (chronoeffectiveness) and tolerance (chronotolerance).of medications. Many pharmacokinetic studies have reported alterations in the concentration of drugs vary as a function of time of administration during the day and on the biological rhythms of the physiological functions [26]. Time-dependent changes in pharmacokinetics may proceed from circadian variations at each step, e.g. absorption, distribution, metabolism and elimination. Therefore, circadian variations in gastric acid secretion and pH, motility, gastric emptying time, gastrointestinal blood flow, drug protein binding, liver enzyme activity and/or hepatic blood flow, glomerular filtration, renal blood flow, urinary pH and tubular resorption may play a role in kinetic variations [27]. Chronopharmacology has been used to optimize the dose of the drug depending upon the time of drug administration in order reduce adverse or undesired effects. Prednisolone, a synthetic corticosteroid is widely used in inflammatory conditions, neoplastic diseases, asthma and as an immunosuppressive agent. Prednisone is both, a prodrug and a metabolite of the active drug Prednisolone. Levonorgestrel and Ethinyl estradiol are widely used as oral contraceptives. In the present study an attempt is made to study the chronopharmacokinetic behavior of Prednisolone, Levonorgestrel and Ethinyl estradiol in humans after dosing at different times of a day.

The method was developed and validated (chapter-4, 5 and 6) were applied for the chronopharmacokinetic study of prednisolone, levonorgestrel and ethinyl estradiol respectively in healthy human subjects. The protocols for the conduction of the study were prepared and approved by the Manipal University Ethics committee. The approval number for the conduct of Prednisolone chronopharmacokinetic study was UEC/18/2008 and that for chronopharmacokinetic study of Ethinyl estradiol/Levonorgestrel was UEC/53/2010. Ethical Committee (EC) approval letters were presented in Appendix-1 and EC approved protocols were presented in the Appendix-2. The pharmacokinetic parameters were calculated using winonlin software, Version, 5.0.1.

8.1 CHRONOPHARMACOKINETIC STUDIES

Two studies were planned to evaluate the chropharmacokinetics of prednisolone, ethinyl estradiol and levonorgestrel [163,164].

8.1.1 Application of the method for chronopharmacokinetic study of Prednisolone

Chropharmacokinetic study of Prednisolone was studied in 12 healthy, human, male volunteers. Volunteers were enrolled into the study as per the inclusion and exclusion criteria mentioned in the protocol. Physical examinations, hematology, biochemical tests, urine analysis and tests for drugs of abuse; all tests were done prior to volunteer enrollment in to the study to assess their eligibility to participate in the study. One tablet of Prednisolone 20mg tablet was administered to the volunteers after high fat high calorie breakfast and the blood samples were collected at the specified time intervals as mentioned in the study procedure. A copy EC approved Informed Consent form (ICF) was given to the volunteers who participated in the study. Schematic diagram of the clinical study is determined in *figure no.29*. The concentrations of Prednisolone and prednisone were estimated using the validated LC-MS/MS method.

From the results obtained, following pharmacokinetic parameters were calculated using Winnonlin Version 5.0.1.

Cmax: Peak concentration, taken as maximum observed concentration in plasma.

tmax: Time to reach peak concentration,

AUC_{0-t}: Area under the concentration-time curve from time zero to the last sample with quantifiable concentration calculated using the linear trapezoidal method.

$t_{½}$: The terminal elimination half-life.

Kel: The elimination rate constant

Study flow chart:

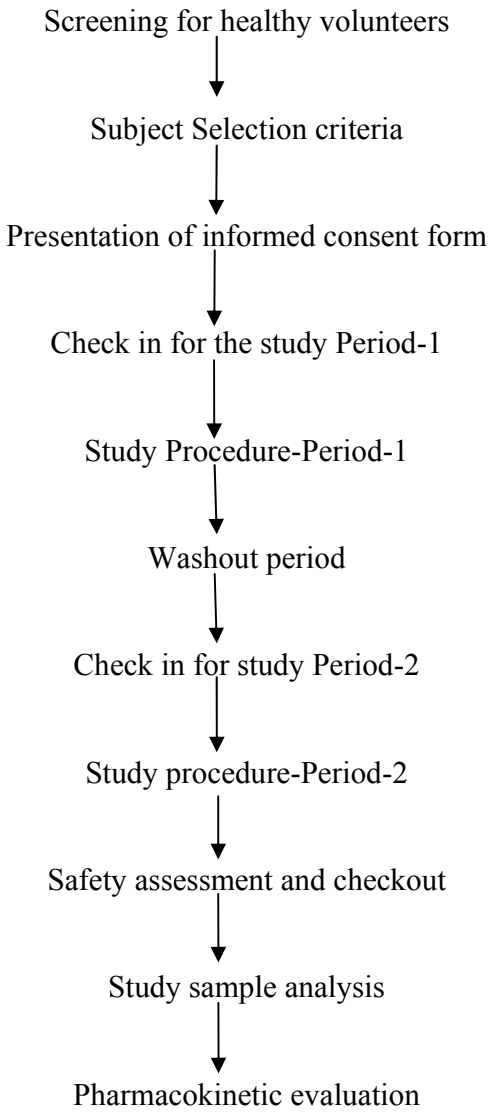

Figure No.29: Schematic diagram of study flow chart

Study-1

An open label, two treatment, two period, two sequence, single dose, bioavailability study of Prednisolone (commercially available) 20 mg tablets in 12 healthy human adult male volunteers under fed conditions.

Study procedure:

Period 1	**Activity**
Day 0 (Pre-Study day)	Test for alcohol and drugs of abuse, Informed Consent Process, volunteer history, examination of vital signs, enquiry about well-being, application of inclusion-exclusion criteria followed by enrollment of volunteers into the study and check-in of the subjects into clinical study facility, catering, and start of 10.00 hours overnight fasting.
Day 1 (Dosing day) Morning Dosing (Dosing at 8:00AM)	Cannulation, pre-dose blood sampling, examination of vital signs, enquiry about well-being, fitness check, high calorie high fat standard breakfast, Dosing at scheduled time and fasting for next 04.00 hours. Blood sample collection, examination of vital signs, enquiry of well-being, catering of study meals at scheduled times and checkout, provided that the 12.00 hours examination of vital signs and enquiry of well-being is satisfactory.
Washout period of at least 07 days calculated from the day of dosing.	
Period 2	**Activity**
Day 0 (Pre-Study day)	Test for alcohol and drugs of abuse, volunteer history, examination of vital signs, enquiry about well-being, check-in of the subjects into clinical study facility, catering, and start of 10.00 hours overnight fasting.
Day 1 (Dosing day) Evening Dosing (Dosing at 8:00PM)	Cannulation, pre-dose blood sampling, examination of vital signs, enquiry about well-being, fitness check, high calorie high fat standard breakfast, Dosing at scheduled time and fasting for next 04.00 hours. Blood sample collection, examination of vital signs, enquiry of well-being, catering of study meals at scheduled times and checkout, provided that the 12.00 hours vital signs examination and enquiry of well-being is satisfactory. After 12.00 hours blood sampling, a blood sample (1×10 mL) collected before check-out for performing post-study safety analysis to assess safety of study subjects. This includes hematology and clinical bio- chemistry.

Study was conducted in 12 healthy male adult healthy volunteers. In each period single oral dose of a 20 mg prednisone was given. In one period volunteers were dosed at 8.00 AM and in another period drug is dosed at 8.00PM. Blood samples (3 mL each) were taken in K_2EDTA vacutainers prior to dosage and at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.50, 6.0, 8.0, and 12.0 and 16.0 hours after dosing. All the blood samples collected in K_2EDTA

vacutainers were centrifuged under refrigeration (8^0C to 10^0C), at 3500 rpm for 10 minutes. The plasma was separated into pre-labelled polypropylene tubes and stored at -70 ± 5^0 C until the completion of study sample analysis. Analysis of the samples was done by using the developed & validated LC-MS/MS method (chapter 4).

The individual plasma concentrations of Prednisolone and Prednisone were measured after dosing at 8.00 AM (Period-1) and 8.00PM (Period-2). The results are presented in *table no.37* and *38 for* Prednisolone and *39 and 40* for prednisone. The pharmacokinetic parameters are presented in *tab*le *No.41 and 42 and c*omparative mean linear plasma concentration v/s time curve is presented in the *figure No. 30 and 31* for Prednisolone and prednisone respectively.

Table No.37. Plasma concentrations of prednisolone in volunteers dosed at 8:00AM (P-1)

Sr.No	Time points	SUB-01	SUB-02	SUB-03	SUB-04	SUB-05	SUB-06	SUB-07	SUB-08	SUB-09	SUB-10	SUB-11	SUB-12
Plasma Concentration(ng/mL)													
1	00.00	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2	00.50	40.862	50.926	45.600	100.318	154.633	125.963	0.000	247.596	115.335	110.398	49.588	116.951
3	01.00	200.056	111.451	130.807	230.979	249.163	311.512	242.682	344.338	306.497	232.639	147.745	212.225
4	01.50	400.557	332.122	322.674	475.689	333.248	398.995	456.928	429.550	400.098	374.652	330.564	410.442
5	02.00	362.355	421.044	417.676	204.165	327.898	395.511	302.810	480.471	467.945	417.839	363.343	398.455
6	02.50	209.892	312.175	210.351	110.003	319.342	286.805	190.768	311.145	396.959	540.987	323.436	297.685
7	03.00	167.003	207.481	104.575	91.393	211.815	288.777	163.928	236.714	289.444	346.746	288.624	191.677
8	03.50	127.037	103.928	78.525	28.916	150.607	162.654	151.940	203.315	273.092	241.540	204.220	170.538
9	04.00	104.846	80.138	66.407	22.609	100.265	101.670	143.285	100.308	218.219	215.174	102.323	155.616
10	04.50	87.955	50.179	51.099	17.028	80.441	88.673	122.244	82.414	161.772	181.597	90.413	146.359
11	05.00	24.554	30.216	51.582	13.866	40.815	63.727	83.490	64.235	137.685	135.630	74.955	120.973
12	05.50	22.346	12.043	32.376	12.278	26.238	32.611	50.910	40.180	125.117	110.228	44.361	129.717
13	06.00	10.510	9.699	26.768	10.156	31.164	25.628	37.326	28.905	118.389	82.783	27.407	56.437
14	08.00	BLQ	7.247	14.770	9.049	19.875	14.589	11.421	14.122	90.352	54.136	18.564	41.784
15	12.00	BLQ	BLQ	9.438	9.443	10.864	8.581	BLQ	10.017	35.596	41.215	10.813	7.995
16	16.00	0.000	0.000	BLQ	BLQ	BLQ	BLQ	0.000	5.141	13.029	7.888	BLQ	BLQ

Table No.38. Plasma concentrations of prednisolone in volunteers dosed at 8:00PM (P-2)

Sr.No	Time points	SUB-01	SUB-02	SUB-03	SUB-04	SUB-05	SUB-06	SUB-07	SUB-08	SUB-09	SUB-10	SUB-11	SUB-12
							Plasma Concentration(ng/mL)						
1	00.00	0.000	0.000	1.006	0.000	1.806	1.002	0.000	0.000	0.000	0.000	0.000	0.000
2	00.50	30.862	30.729	61.600	80.018	55.653	95.903	0.632	67.596	85.335	70.398	38.560	56.788
3	01.00	100.056	91.421	120.807	130.879	144.103	250.514	52.682	124.338	106.736	132.639	168.456	123.566
4	01.50	300.557	232.222	222.634	375.609	235.028	299.095	156.928	329.550	256.697	274.652	230.567	230.566
5	02.00	252.245	391.044	397.206	304.102	297.088	268.211	202.810	360.471	345.340	317.539	268.567	345.677
6	02.50	309.762	362.195	410.351	210.003	419.022	286.405	390.768	310.145	394.959	440.987	345.567	489.567
7	03.00	267.903	407.411	404.575	102.393	311.802	288.797	267.500	206.744	256.454	376.460	389.546	291.670
8	03.50	227.137	300.524	230.425	98.206	250.602	262.154	261.460	183.310	373.520	341.940	234.567	270.456
9	04.00	204.826	100.128	126.307	52.600	150.261	201.470	243.685	120.368	218.259	315.774	126.567	176.456
10	04.50	167.555	115.179	151.029	37.008	100.401	188.073	167.564	98.404	171.250	281.897	100.566	134.673
11	05.00	114.534	80.216	21.182	23.860	60.025	56.697	183.459	84.635	145.465	184.670	98.456	90.657
12	05.50	62.326	52.133	34.106	22.208	36.230	28.631	191.000	50.145	131.470	98.463	67.477	110.388
13	06.00	40.523	49.509	16.708	30.056	21.104	15.628	127.356	38.924	68.350	95.677	47.567	66.672
14	08.00	34.515	27.107	15.710	19.000	29.845	24.589	111.201	24.122	39.352	67.660	38.566	46.421
15	12.00	12.326	15.106	19.208	16.083	11.564	18.561	63.690	20.017	15.570	38.758	23.578	12.456
16	16.00	8.631	14.476	8.359	9.831	6.507	8.458	42.194	BLQ	0.000	18.567	8.356	BLQ

BLQ: Below the Limit of Quantification

Table No.39. Plasma concentrations of prednisone in volunteers dosed at 8:00AM (P-1)

Sr.No	Time points	SUB-01	SUB-02	SUB-03	SUB-04	SUB-05	SUB-06	SUB-07	SUB-08	SUB-09	SUB-10	SUB-11	SUB-12
							Plasma Concentration(ng/mL)						
1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2	0.000	5.926	45.600	18.318	24.633	25.963	0.000	27.596	15.335	11.398	49.588	16.951	0.000
3	10.000	11.451	50.807	30.979	49.163	31.512	42.682	34.338	36.497	23.639	47.745	21.225	10.000
4	40.557	32.122	72.674	55.689	43.248	38.995	56.928	42.550	40.098	37.652	50.564	41.442	40.557
5	62.355	42.044	77.676	64.165	57.898	39.511	82.810	48.471	67.945	41.839	63.343	58.455	62.355
6	59.892	62.175	80.351	70.003	69.342	86.805	90.768	71.145	96.959	54.987	73.436	67.685	59.892
7	96.000	77.481	84.575	91.393	71.815	88.777	63.928	86.714	89.444	66.746	88.624	91.677	96.000
8	82.000	103.928	88.525	58.916	50.607	92.654	51.940	83.315	73.092	71.540	104.220	70.538	82.000
9	74.846	80.138	66.407	32.609	30.265	101.670	43.285	100.308	48.219	85.174	92.323	45.616	74.846
10	77.955	50.179	51.099	17.028	20.441	88.673	32.244	82.414	31.772	81.597	90.413	46.359	77.955
11	64.554	30.216	51.582	13.866	10.815	63.727	23.490	64.235	27.685	65.630	74.955	40.973	64.554
12	42.346	12.043	32.376	12.278	6.238	32.611	10.910	40.180	25.117	50.228	44.361	29.717	42.346
13	30.510	9.699	26.768	10.156	7.164	25.628	7.326	28.905	18.389	32.783	27.407	16.437	30.510
14	14.915	7.247	14.770	9.049	9.875	14.589	8.421	14.122	10.352	24.136	18.564	11.784	14.915
15	8.346	BLQ	9.438	9.443	5.864	8.581	BLQ	10.017	5.596	11.215	10.813	7.995	8.346
16	5.631	BLQ	BLQ	0.000	BLQ	BLQ	BLQ	5.141	0.000	7.888	BLQ	BLQ	5.631

Table No.40. Plasma concentrations of prednisone in volunteers dosed at 8:00PM (P-2)

Sr.No	Time points	SUB-01	SUB-02	SUB-03	SUB-04	SUB-05	SUB-06	SUB-07	SUB-08	SUB-09	SUB-10	SUB-11	SUB-12
Plasma Concentration(ng/mL)													
1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2	0.000	4.345	34.240	25.650	14.545	15.344	18.636	38.567	25.367	23.467	49.588	12.578	0.000
3	5.000	23.456	45.526	35.765	33.256	26.535	56.355	42.570	87.355	45.789	47.745	34.880	5.000
4	36.788	45.354	56.876	67.723	43.757	46.535	67.245	42.550	92.646	67.246	50.564	38.266	36.788
5	46.677	38.646	78.455	75.664	58.456	58.355	78.245	34.366	104.664	76.270	63.343	43.677	46.677
6	65.677	52.564	76.426	80.536	67.000	76.423	86.245	64.882	85.455	67.254	73.436	57.366	65.677
7	67.664	67.543	56.523	88.647	83.255	78.566	89.245	78.535	67.455	82.000	87.250	65.355	67.664
8	74.000	85.435	45.620	67.535	73.883	86.536	92.435	79.245	54.378	87.000	90.276	78.255	74.000
9	78.355	76.358	34.727	56.366	68.455	90.635	68.355	80.256	38.546	85.174	87.715	80.455	78.355
10	80.546	60.452	24.767	43.757	56.366	100.646	54.344	87.525	24.758	81.597	67.154	64.525	80.546
11	84.884	22.657	20.625	30.626	43.424	86.563	45.355	93.657	14.476	65.630	55.762	56.244	84.884
12	64.000	8.563	12.660	26.525	36.455	76.536	36.424	67.456	12.567	50.228	32.747	30.244	64.000
13	37.647	5.564	16.656	16.563	23.545	56.355	24.244	56.828	8.345	32.783	12.466	27.453	37.647
14	27.563	4.646	8.672	12.758	10.355	35.366	14.356	49.466	4.973	24.136	10.455	12.560	27.563
15	17.456	3.757	5.652	8.524	8.545	28.466	8.345	32.588	3.861	11.215	8.435	9.553	17.456
16	12.456	1.569	3.672	6.546	6.345	10.667	5.345	12.774	0.000	7.888	6.156	6.616	12.456

BLQ: Below the Limit of Quantification

Table No.41. Comparative pharmacokinetic parameters of Prednisolone

COMPARATIVE PHARMACOKINETIC PARAMETERS												
Subject	t1/2 (hr)		λz (1/hr)		tmax (hr)		Cmax (ng/mL)		AUC_T (ng.h/mL)		AUC_I (ng.h/mL)	
	Period		Period		Period		Period		Period		Period	
	1	2	1	2	1	2	1	2	1	2	1	2
1	0.878	3.774	0.789	0.184	1.50	2.50	400.557	309.762	876.359	1239.646	889.6759	1286.6411
2	0.891	5.335	0.778	0.130	2.00	3.00	421.044	407.411	875.222	1314.184	884.5352	1425.6087
3	2.550	2.592	0.272	0.267	2.00	2.50	417.676	410.351	852.482	1251.927	887.1973	1283.1821
4	1.953	6.887	0.355	0.101	1.50	1.50	475.689	375.609	712.350	897.007	738.9553	994.6918
5	4.027	3.641	0.172	0.190	1.50	2.50	333.248	419.022	1117.541	1205.744	1180.6608	1239.9208
6	3.521	5.196	0.197	0.133	1.50	1.50	398.995	299.095	1221.413	1297.687	1264.9960	1361.0916
7	1.163	6.035	0.596	0.115	1.50	2.50	456.928	390.768	1012.571	1890.874	1031.7271	2258.2539
8	5.488	4.515	0.126	0.154	2.00	2.00	480.471	360.471	1398.980	1185.224	1439.6814	1238.2506
9	2.863	2.033	0.242	0.341	2.00	2.50	467.945	394.959	2033.566	1514.674	2087.3897	1523.7034
10	2.796	4.377	0.248	0.158	2.50	2.50	540.987	440.987	1900.238	1932.452	1932.0516	2049.6870
11	4.555	3.872	0.152	0.179	2.00	3.00	363.343	389.546	1121.363	1321.243	1192.4229	1367.9176
12	1.867	2.177	0.371	0.318	1.50	2.50	410.442	489.567	1387.207	1439.927	1408.7398	1451.3393
N	12	12	12	12	12	12	12	12	12	12	12	12
Mean	2.713	4.203	0.358	0.189	1.792	2.375	430.610	390.629	1209.108	1374.216	1244.836	1456.691
SD	1.4744	1.5070	0.2353	0.0788	0.3343	0.4827	56.6972	52.1139	412.9909	292.2901	419.8977	354.6860
Minimum	0.878	2.033	0.126	0.101	1.500	1.500	333.248	299.095	712.350	897.007	738.955	994.692
Median	2.673	4.124	0.260	0.169	1.750	2.500	419.360	392.863	1119.452	1305.936	1186.542	1364.505
Maximum	5.488	6.887	0.789	0.341	2.500	3.000	540.987	489.567	2033.566	1932.452	2087.390	2258.254
CV%	54.35	35.86	65.67	41.62	18.66	20.32	13.17	13.34	34.16	21.27	33.73	24.35
Geometric Mean	2.317	3.938	0.299	0.176	1.765	2.323	427.182	387.342	1151.131	1347.144	1186.113	1422.251

Table No.42. Comparative pharmacokinetic parameters of Prednisone

COMPARATIVE PHARMACOKINETIC PARAMETERS												
Subject	t1/2 (hr)		λz (1/hr)		tmax (hr)		Cmax (ng/mL)		AUC_T (ng.h/mL)		AUC_I (ng.h/mL)	
	Period		Period		Period		Period		Period		Period	
	1	2	1	2	1	2	1	2	1	2	1	2
1	5.693	6.981	0.122	0.099	3.00	5.00	96.000	84.884	432.781	526.279	479.0277	651.7376
2	3.712	5.135	0.187	0.135	3.50	3.50	103.928	85.435	273.222	281.745	312.0288	293.3688
3	3.761	6.453	0.184	0.107	3.50	2.00	88.525	78.455	447.482	320.010	498.6933	354.1940
4	3.183	7.504	0.218	0.092	3.00	3.00	91.393	88.647	291.350	405.563	334.7075	476.4324
5	2.834	11.321	0.245	0.061	3.00	3.00	71.815	83.255	267.541	396.792	291.5202	500.4259
6	3.521	3.876	0.197	0.179	4.00	4.50	101.670	100.646	438.413	683.077	481.9960	742.7179
7	1.375	5.612	0.504	0.124	2.50	3.50	90.768	92.435	267.071	463.885	283.7742	507.1641
8	5.488	4.733	0.126	0.146	4.00	5.00	100.308	93.657	469.480	730.138	510.1814	817.3592
9	2.945	1.879	0.235	0.369	2.50	2.00	96.959	104.664	341.316	336.906	365.0885	347.3702
10	4.712	4.712	0.147	0.147	4.00	3.50	85.174	87.000	469.238	539.850	522.8630	593.4755
11	4.555	10.156	0.152	0.068	3.50	3.50	104.220	90.276	501.363	445.789	572.4229	535.9864
12	5.934	8.650	0.117	0.080	3.00	4.00	91.677	80.455	337.207	404.363	405.6492	486.9304
N	12	12	12	12	12	12	12	12	12	12	12	12
Mean	3.976	6.418	0.203	0.134	3.292	3.542	93.536	89.151	378.039	461.200	421.496	525.597
SD	1.3493	2.6902	0.1044	0.0819	0.5418	0.9876	9.2171	7.7859	90.1477	138.2634	101.2010	157.4954
Minimum	1.375	1.879	0.117	0.061	2.500	2.000	71.815	78.455	267.071	281.745	283.774	293.369
Median	3.736	6.033	0.186	0.115	3.250	3.500	93.839	87.823	387.048	425.676	442.338	503.795
Maximum	5.934	11.321	0.504	0.369	4.000	5.000	104.220	104.664	501.363	730.138	572.423	817.359
CV%	33.94	41.92	51.49	61.12	16.46	27.89	9.85	8.73	23.85	29.98	24.01	29.97
Geometric Mean	3.725	5.840	0.186	0.119	3.250	3.405	93.089	88.848	367.853	443.509	409.899	503.817

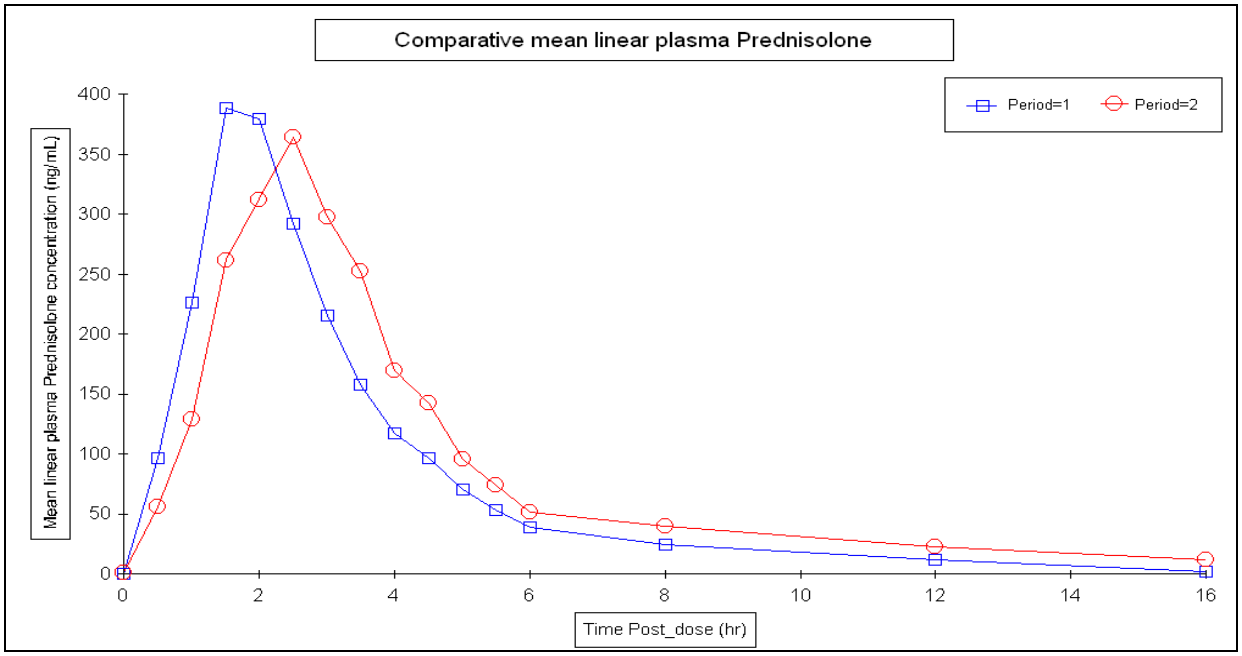

Figure No.30. Mean linear plasma concentration of Prednisolone (time v/s mean plasma conc.)

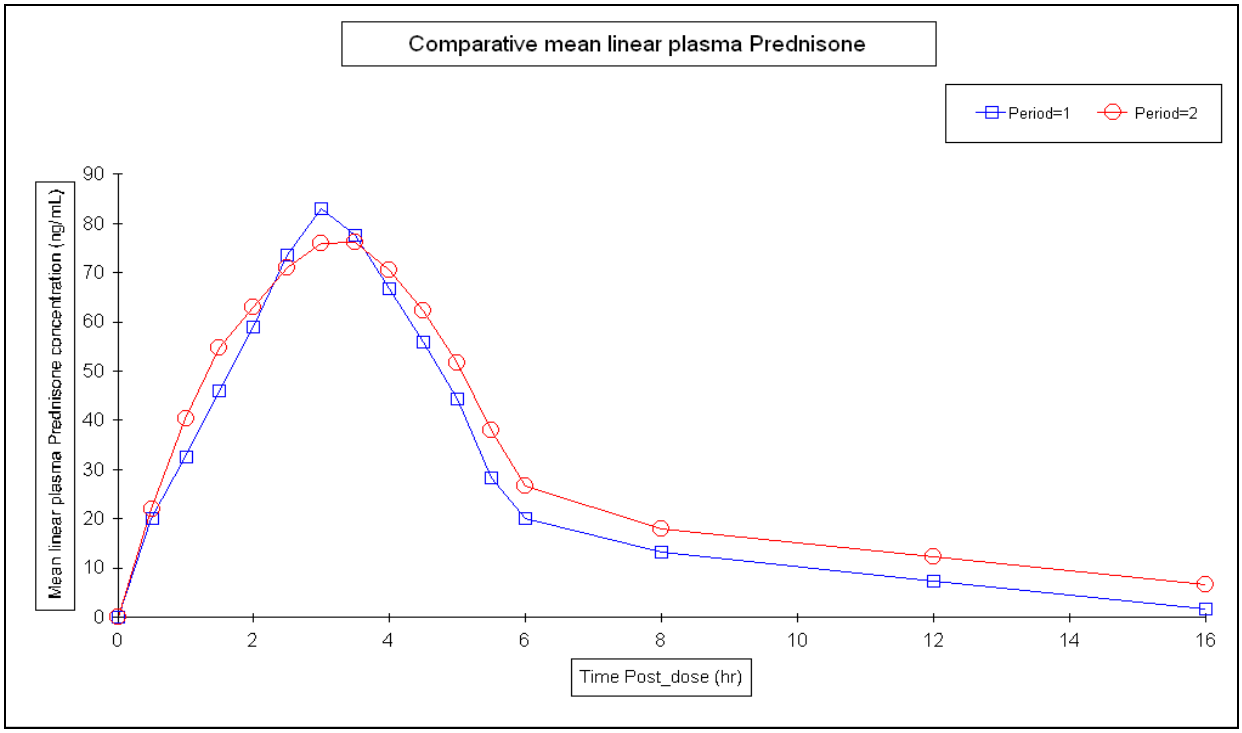

Figure No.31. Mean linear plasma concentration of Prednisone (time v/s mean plasma conc.)

CONCLUSION:

The application of systemic and oral corticosteroids has been used successfully for many years for a wide variety of inflammatory disorders and for immunosuppressive effects. Typically the dosage regimen is highly individualized, with the goal of obtaining the maximal benefit with minimal risk of adverse effects. Many patients with asthma are prescribed corticosteroids for the control the of the signs and symptoms of chronic asthma. This needs a short course of high-dose oral prednisone or oral glucocorticoids in addition to inhaled corticosteroids [36].

Pharmacokinetic parameters of Prednisolone have shown more C_{max} during day time dosing when compared to night time (430.610ng/ml and 390.629ng/mL), but the values are not clinically significance. C_{max} of Prednisone was found to be 93.536 ng/mL during the day time and 89.151ng/mL during night time dosing. Variation in half-life $t_{1/2}$ was observed for Prednisolone was 2.713 to 4.203 hr and for Prednisone it was 3.976 to 6.418 hr. Time to reach the maximum concentration $t_{max,}$ varied about 1.5 times (1.792 to 2.375 hr) for Prednisolone but there was no significant difference with prednisone (3.292hr to 3.542hr). The results have

shown the area under curve (AUC_{inf}) to be greater during the night time dosing than day time dosing for Prednisolone (1244.836ng.h/mL and 1456.691ng.h/mL) and prednisone was (421.496. ng.h/mL and 525.597ng.h/mL) The extent of variation in Prednisolone absorption is determined by Tnax of AUC_{inf} and showed an intra subject variation of 33.73% and a variation of 20.32% for t_{max} of Prednisolone. Prednisolone is metabolized by hepatic enzymes as shown in the litratures. [26]. The degree of hepatic clearance of orally dosed drugs depends on the hepatic excretion ratio. There are reports stating that the hepatic enzymes show chonobiological variations in their activity [156]. There are considerable evidences that the food also alters the bioavailability of the drug [149].Food alters the drug absorption due to degradation of the drug, complexation of the drug with the components of the food, reduced dissolution due to the presence of the food and also altered gastro intestinal motility. In the present study the drug was dosed in fed conditions and the bioavailability of the drug is delayed during night time dosing. There was a significant delay in the time to achieve the maximum concentration of the drug and the concentration achieved is also more with night time dosing when compared to day time dosing. Blood flow rate, gastro intestinal motility and emptying rate are important factors that influence the absorption of the drug. The rate of drug diffusion across the gut capillary membrane is a function of concentration gradient across the membrane and the rate of blood flow through the capillary membranes. The presence of food brings about the alterations in gastro intestinal motility and blood flow. This may have an impact on the absorption of the drug.

The results achieved in the present study have shown that elimination rate constant was more during night time dosing when compared to day time dosing 0.358 and 0.189 respectively. Since Prednisolone is metabolized by the hepatic enzymes, increase in hepatic blood results in increased elimination of Prednisolone.

The study has shown temporal differences in drug elimination rate constant when the drug administered morning and evening dosing. Time related differences in drug effects depend upon endogenous circadian rhythms, which include metabolic pathways.

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8.2 Application of the method for chronopharmacokinetic study of Levonorgestrel and Ethinyl estradiol

Treatment with low dose Levonorgestrel has emerged as effective, convenient and safe method for emergency contraception. The most widely used emergency contraception methods in the world are the Yuzpe regimen (combined estrogen–progestin contraceptive pills) and the Levonorgestrel regimen (progestin only) [118]

Ethinyl estradiol is the estrogenic component of most combined oral contraceptive preparations. Hydroxylation to the catechol 2-hydroxyethinyl estradiol is a principal route of metabolism in animals and humans. In addition conjugation with glucuronic acid and sulphate are important metabolic pathways with the conjugates excreted in bile and urine [119].

Study-2

A open label, two period, single dose, chronopharmacokinetic study of commercially available Ethinylestradiol and Levonorgestrel(0.05mg /0.25mg) tablets in 12 healthy human adult female subjects under fasting conditions.

Study procedure:

Period 1	**Activity**
Day 0 (Pre-Study day)	Urine pregnancy test for alcohol and drugs of abuse, Informed Consent Process, volunteer history, examination of vital signs, enquiry about well-being, application of inclusion-exclusion criteria followed by enrollment of volunteers into the study and check-in of the subjects into clinical study facility, catering, and start of 10.00 hours overnight fasting.
Day 1 (Dosing day) Morning Dosing (8:00AM)	Cannulation, pre-dose blood sampling, examination of vital signs, enquiry about well-being, fitness check, allocated dose of IP given at scheduled time and continued fasting for next 04.00 hours. Blood sample collection, examination of vital signs, enquiry of well-being, catering of study meals at scheduled times and checkout, provided that the 24.00 hours vital signs examination and enquiry of well-being is satisfactory
Ambulatory samples	48.00 and 72.00 hours after dosing
Washout period of at least 14 days calculated from the day of dosing.	

Period 2	Activity
Day 0 (Pre-Study day)	Urine pregnancy test, Test for alcohol and drugs of abuse, volunteer history, examination of vital signs, and enquiry about well-being, check-in of the subjects into clinical study facility, catering, and start of 10.00 hours overnight fasting.
Day 1 (Dosing day) Evening Dosing (8:00PM)	Cannulation, pre-dose blood sampling, examination of vital signs, enquiry about well-being, fitness check, dosing at scheduled time and continued fasting for next 04.00 hours. Blood sample collection, vital signs examination, enquiry of well-being, catering of study meals at scheduled times and checkout, provided that the 24.00 hours, examination of vital signs and enquiry of well-being is satisfactory
Ambulatory samples	48.00 and 72.00 hrs after dosing
Safety samples	After 72.00 hours blood sampling, blood samples (2×4 mL) collected for performing post-study safety analysis to assess safety of study subjects. This includes hematology, bio chemistry and urine pregnancy test.

Procedure:

The study was conducted with 12 healthy female adult volunteers. In each period of the study single oral dose of commercially available Ethinylestradiol and Levonorgestrel (0.05mg /0.25mg) tablet was administered orally. In one period volunteers were dosed at 8.00 AM and in another period drug was administered at 8.00PM. A total of 19 (1×4.0 mL) blood samples were collected from each subject in K_2EDTA vacutainers in each study period. The pre-dose sample (1×4.0 mL) was collected within 01.00 hour before dosing in each study period. The post-dose samples (1×4.0mL) were collected at 00.25, 00.50, 00.75, 01.00, 01.25, 01.50, 01.75, 02.00, 02.33, 02.67, 03.00, 03.50, 04.00, 08.00, 16.00, 24.00, 48.00 and 72.00 hours after dosing in each study period. After collection, the blood samples were centrifuged at 3500 rpm under refrigeration at 4°C for 5 min. After centrifugation, the separated plasma was transferred into suitably labeled polypropylene tubes. All plasma samples were stored upright in a deep freezer set at –70°C ± 5°C until the completion of study sample analysis. Analysis of the samples was done by using validated LC-MS/MS method reported in chapters 5 and 6.

Volunteers were enrolled into the study as per the inclusion and exclusion criteria mentioned in the protocol. A single tablet was dosed to the volunteers at the predetermined time and the blood samples were collected at the specified time intervals. The individual plasma

concentrations and comparative pharmacokinetic parameters of Levonorgestrel are presented in the *table No.43, 44 and 45*. The individual plasma concentrations and comparative pharmacokinetic parameters of Ethinyl estradiol are presented in the *table No.46, 47 and 48*. Mean plasma concentration v/s time curves are presented in *figure No. 32 and 33* for Levonorgestrel and Ethinyl estradiol respectively.

Table No.43. Plasma concentrations of Levonorgestrel in volunteers dosed at 8:00AM (Period-1)

Sr.No	Time points	SUB-01	SUB-02	SUB-03	SUB-04	SUB-05	SUB-06	SUB-07	SUB-08	SUB-09	SUB-10	SUB-11	SUB-12
							Plasma Concentration(ng/mL)						
1	0.00	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2	0.25	1.023	1.768	1.856	1.435	0.958	0.995	1.032	1.057	1.230	1.089	1.690	1.769
3	0.50	2.953	3.034	3.787	2.455	1.457	1.988	1.993	2.076	2.567	2.087	2.877	2.043
4	0.75	4.253	3.789	4.050	2.957	2.574	2.088	2.653	2.895	3.234	2.876	3.020	2.780
5	1.00	4.562	4.094	4.995	3.560	2.897	2.598	3.546	3.250	3.739	3.056	3.680	2.540
6	1.25	5.023	4.563	5.096	4.090	3.043	3.895	4.054	3.786	4.036	3.678	4.130	3.765
7	1.50	4.024	4.967	5.990	4.345	3.547	4.940	4.554	4.046	5.067	4.065	4.784	4.030
8	1.75	3.625	5.067	5.443	4.879	4.024	5.040	5.056	4.563	5.540	5.068	5.230	4.530
9	2.00	3.925	5.679	5.045	5.020	4.890	5.400	5.510	4.044	5.530	5.554	5.660	5.520
10	2.33	3.254	5.067	4.676	5.123	4.956	5.130	5.349	4.580	4.897	5.079	5.010	5.872
11	2.67	3.025	5.021	4.353	6.124	4.712	4.768	6.020	5.520	4.545	4.657	4.879	5.435
12	3.00	2.920	4.780	4.070	5.021	4.925	4.345	4.945	5.050	4.290	4.231	5.430	4.040
13	3.50	2.602	4.340	3.678	3.435	5.023	4.130	4.645	4.879	3.878	3.668	4.392	3.674
14	4.00	2.001	3.956	3.050	3.231	3.456	3.968	4.520	4.456	3.536	3.087	4.090	3.213
15	8.00	1.632	2.010	2.450	2.678	3.093	3.457	3.897	3.876	3.320	2.055	3.453	3.023
16	16.00	1.203	1.879	2.230	2.340	2.785	3.070	2.994	3.453	3.030	1.546	2.655	2.562
17	24.00	0.802	0.909	0.804	0.986	2.095	2.897	2.346	2.500	2.789	1.432	1.567	2.140
18	48.00	0.202	0.409	0.305	0.308	0.897	1.578	1.575	1.675	1.394	1.083	1.045	1.342
19	72.00	0.102	0.107	0.145	0.109	0.546	0.647	0.854	0.366	0.454	0.564	0.734	0.994

Table No.44. Plasma concentrations of Levonorgestrel in volunteers dosed at 8:00PM (Period-2)

Sr.No	Time points	SUB-01	SUB-02	SUB-03	SUB-04	SUB-05	SUB-06	SUB-07	SUB-08	SUB-09	SUB-10	SUB-11	SUB-12
Plasma Concentration(ng/mL)													
1	0.00	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2	0.25	0.925	0.765	1.094	1.998	1.093	0.988	0.568	0.795	0.988	0.888	0.966	0.987
3	0.50	1.923	1.234	2.935	2.546	3.767	1.972	1.455	3.087	3.124	3.065	2.096	1.999
4	0.75	2.036	3.023	4.989	2.988	4.984	2.056	2.568	3.540	3.567	3.879	3.434	2.658
5	1.00	3.025	3.984	5.053	3.974	5.233	3.093	3.443	4.960	4.898	4.068	4.093	3.098
6	1.25	4.024	3.763	5.998	4.342	5.560	4.035	4.787	4.345	5.056	4.668	4.090	4.537
7	1.50	5.023	4.245	4.099	4.778	5.300	5.046	5.234	5.098	5.550	4.890	4.897	5.987
8	1.75	5.632	5.981	3.453	4.890	4.679	5.899	5.440	5.570	4.987	5.150	4.435	5.099
9	2.00	5.426	5.433	3.342	4.430	4.234	5.435	5.580	5.430	4.604	5.580	4.535	4.769
10	2.33	5.102	5.093	3.123	3.653	4.098	4.094	5.090	5.054	4.389	5.070	5.124	4.345
11	2.67	4.980	4.939	3.090	3.342	3.086	3.999	4.897	4.987	4.309	5.987	3.092	4.092
12	3.00	4.625	4.042	2.655	3.984	2.998	3.562	4.345	4.455	3.786	3.435	2.980	3.986
13	3.50	4.412	3.988	2.342	3.565	2.786	3.324	4.098	4.025	3.456	4.345	2.342	3.522
14	4.00	4.012	3.932	2.232	3.343	2.547	3.098	3.325	3.044	3.320	3.321	2.190	3.213
15	8.00	3.021	2.939	1.837	2.786	2.345	2.678	3.234	2.990	2.897	3.120	1.998	3.009
16	16.00	2.201	2.021	1.673	2.345	1.897	2.056	3.098	2.450	2.453	3.677	1.456	2.658
17	24.00	1.250	1.828	1.429	1.865	1.453	1.989	2.098	2.098	2.210	2.872	1.298	2.095
18	48.00	0.963	0.879	0.769	0.986	1.005	0.987	1.098	1.097	1.231	1.949	0.989	1.065
19	72.00	0.301	0.487	0.345	0.465	0.769	0.985	0.879	0.988	0.856	0.564	0.425	0.984

Table No.45. Comparative pharmacokinetic parameters of Levonorgestrel

COMPARATIVE PHARMACOKINETIC PARAMETERS												
Subject	t1/2 (hr)		λ_z (1/hr)		tmax (hr)		Cmax (ng/mL)		AUC_T (ng.h/mL)		AUC_I (ng.h/mL)	
	Period		Period		Period		Period		Period		Period	
	1	2	1	2	1	2	1	2	1	2	1	2
1	15.183	18.071	0.046	0.038	1.25	1.75	5.023	5.632	54.943	106.208	57.1767	114.0559
2	14.979	25.154	0.046	0.028	2.00	1.75	5.679	5.981	77.638	113.193	79.9499	130.8662
3	19.424	24.546	0.036	0.028	1.50	1.25	5.990	5.998	77.147	87.117	81.2099	99.3338
4	15.107	24.178	0.046	0.029	2.67	1.75	6.124	4.890	81.489	115.525	83.8644	131.7447
5	24.371	52.289	0.028	0.013	3.50	1.25	5.023	5.560	124.051	105.498	143.2483	163.5093
6	22.194	35.603	0.031	0.019	2.00	1.75	5.400	5.899	160.354	120.018	181.0706	170.6116
7	32.163	30.648	0.022	0.023	2.67	2.00	6.020	5.580	158.591	136.827	198.2177	175.6932
8	20.234	37.790	0.034	0.018	2.67	1.75	5.520	5.570	160.008	131.790	170.6919	185.6541
9	18.328	34.667	0.038	0.020	1.75	1.50	5.540	5.550	150.483	134.243	162.4877	177.0542
10	37.674	21.775	0.018	0.032	2.00	2.67	5.554	5.987	101.258	170.677	131.9121	188.3943
11	43.870	30.633	0.016	0.023	2.00	2.33	5.660	5.124	125.877	90.645	172.3320	109.4277
12	38.979	34.967	0.018	0.020	2.33	1.50	5.872	5.987	138.506	131.151	194.4026	180.7910
N	12	12	12	12	12	12	12	12	12	12	12	12
Mean	25.209	30.860	0.032	0.024	2.195	1.771	5.617	5.646	117.529	120.241	138.047	152.261
SD	10.2873	9.1668	0.0113	0.0069	0.6123	0.4118	0.3569	0.3572	37.7378	22.8167	50.1200	32.8024
Minimum	14.979	18.071	0.016	0.013	1.250	1.250	5.023	4.890	54.943	87.117	57.177	99.334
Median	21.214	30.640	0.033	0.023	2.000	1.750	5.607	5.606	124.964	117.771	152.868	167.060
Maximum	43.870	52.289	0.046	0.038	3.500	2.670	6.124	5.998	160.354	170.677	198.218	188.394
CV%	40.81	29.70	35.81	28.50	27.90	23.25	6.35	6.33	32.11	18.98	36.31	21.54
Geometric Mean	23.456	29.696	0.030	0.023	2.118	1.730	5.607	5.636	111.255	118.315	128.152	148.744

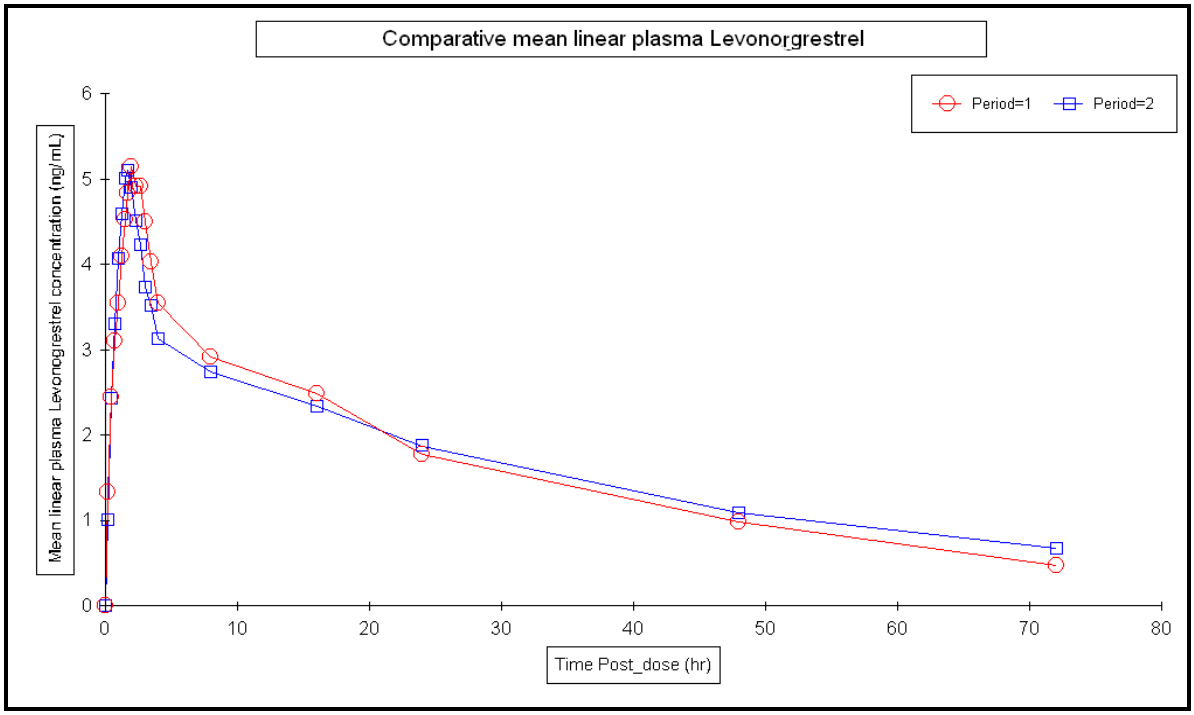

Figure No.32. Mean linear plasma concentration of Levonorgestrel (time v/s mean plasma conc.)

Table No.46. Plasma concentrations of Ethinyl estradiol in volunteers dosed at 8:00AM (P-1)

Sr.No	Time points	SUB-01	SUB-02	SUB-03	SUB-04	SUB-05	SUB-06	SUB-07	SUB-08	SUB-09	SUB-10	SUB-11	SUB-12
						Plasma Concentration(pg/mL)							
1	0.00	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2	0.25	24.025	24.120	25.980	23.050	26.393	25.980	21.342	24.090	22.025	20.746	21.985	19.984
3	0.50	81.256	80.246	79.980	70.240	60.879	79.240	29.800	60.080	45.876	37.870	40.310	30.928
4	0.75	105.023	106.090	102.980	110.300	103.340	107.430	65.345	99.876	81.678	50.790	78.900	50.987
5	1.00	132.025	132.420	104.300	121.490	125.435	140.900	98.780	121.090	99.657	70.466	128.625	83.240
6	1.25	145.023	144.091	112.880	136.900	138.900	144.098	121.900	124.340	124.300	99.460	140.879	114.270
7	1.50	125.362	126.240	136.300	150.860	145.430	123.230	136.000	133.420	138.560	122.980	155.534	129.870
8	1.75	110.235	112.080	123.900	147.800	156.887	112.343	114.980	143.900	140.670	130.680	134.030	149.243
9	2.00	99.212	99.245	124.660	142.434	146.990	89.098	78.945	146.780	132.400	147.987	123.000	130.898
10	2.33	90.256	91.890	122.090	134.980	129.023	82.830	70.987	131.560	123.980	121.679	87.098	112.920
11	2.67	60.256	62.980	100.300	120.654	115.150	65.980	40.650	122.900	115.230	112.090	73.765	99.673
12	3.00	58.024	56.090	84.546	112.980	99.460	51.230	32.434	99.980	81.324	100.786	59.869	76.763
13	3.50	52.015	54.090	80.807	109.090	83.230	43.123	24.450	78.560	75.667	86.987	35.676	60.938
14	4.00	41.025	43.260	71.980	100.430	74.343	25.870	19.567	60.867	60.560	76.093	25.986	40.827
15	8.00	32.045	33.320	54.908	65.540	54.980	18.720	10.576	34.980	29.897	43.534	16.945	24.829
16	16.00	12.062	13.098	32.670	35.867	27.090	13.650	8.213	21.230	14.650	21.022	8.786	12.354
17	24.00	8.024	10.980	15.980	16.987	10.900	7.780	3.456	10.980	9.789	10.987	5.986	6.976
18	48.00	4.032	6.099	9.980	7.987	5.230	3.451	1.211	8.976	4.655	5.098	3.947	1.984
19	72.00	0.853	0.987	1.768	1.052	0.877	0.478	0.543	0.977	1.010	0.923	0.998	0.879

Table No.47. Plasma concentrations of Ethinyl estradiol in volunteers dosed at 8:00PM (P-2)

Sr.No	Time points	SUB-01	SUB-02	SUB-03	SUB-04	SUB-05	SUB-06	SUB-07	SUB-08	SUB-09	SUB-10	SUB-11	SUB-12
Plasma Concentration(pg/mL)													
1	0.00	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
2	0.25	22.035	24.826	25.988	21.870	19.098	23.098	30.654	22.564	28.970	25.838	20.500	26.970
3	0.50	78.024	69.982	53.970	46.870	34.980	75.090	60.826	50.938	39.983	40.824	34.938	44.250
4	0.75	110.025	99.839	82.098	78.980	67.987	115.980	99.873	87.938	60.973	87.300	96.964	78.250
5	1.00	126.035	122.278	118.980	125.250	129.670	128.089	124.928	136.955	76.309	99.938	115.340	121.520
6	1.25	130.024	134.837	132.760	143.980	143.890	130.433	145.300	149.848	123.940	136.930	138.670	144.236
7	1.50	138.023	145.940	142.980	155.870	132.098	139.756	134.035	125.938	146.948	141.287	136.500	145.560
8	1.75	160.204	142.810	150.566	134.980	124.978	159.987	123.435	79.039	123.970	134.920	155.450	125.326
9	2.00	142.031	132.910	134.956	116.235	96.540	139.984	112.924	56.947	116.737	119.928	134.920	99.125
10	2.33	110.023	100.987	123.756	96.237	67.987	112.973	87.029	43.646	76.940	78.980	89.780	78.215
11	2.67	90.021	80.933	110.534	61.235	44.647	94.929	60.974	34.635	40.763	57.970	60.762	55.894
12	3.00	82.031	78.936	97.978	45.236	30.838	79.766	45.094	24.636	23.763	32.970	41.423	34.023
13	3.50	80.024	71.928	60.947	32.450	19.837	75.928	30.756	18.829	19.929	21.980	22.928	21.365
14	4.00	56.032	44.268	41.968	23.236	16.938	45.872	21.636	13.983	12.933	16.876	10.828	15.265
15	8.00	29.024	23.029	32.434	14.260	10.764	24.982	14.978	9.873	7.653	12.938	7.929	10.120
16	16.00	10.025	9.874	8.959	11.254	7.637	10.929	8.987	6.837	3.937	8.929	5.325	8.127
17	24.00	5.023	5.929	4.095	6.250	4.977	4.094	4.974	2.929	1.929	4.653	2.828	3.215
18	48.00	2.032	1.929	3.848	2.155	1.933	2.098	3.083	1.029	0.985	2.564	1.442	2.125
19	72.00	0.433	0.457	1.095	0.658	0.987	0.678	0.987	0.928	0.388	0.876	0.624	0.236

Table No.48. Comparative pharmacokinetic parameters of Ethinyl estradiol

COMPARATIVE PHARMACOKINETIC PARAMETERS												
Subject	t1/2 (hr)		λz (1/hr)		tmax (hr)		Cmax (pg/mL)		AUC_T (pg.h/mL)		AUC_I (pg.h/mL)	
	Period		Period		Period		Period		Period		Period	
	1	2	1	2	1	2	1	2	1	2	1	2
1	14.726	21.031	0.047	0.033	1.75	1.25	160.204	145.023	861.507	867.858	904.6779	990.1942
2	8.173	28.849	0.085	0.024	1.50	1.25	145.940	144.091	696.889	968.194	766.8013	1222.0351
3	8.100	14.012	0.086	0.049	1.75	1.50	150.566	136.300	829.051	1485.925	874.0162	1687.6645
4	14.291	11.241	0.049	0.062	1.50	1.50	155.870	150.860	1001.165	1685.781	845.596	1815.3063
5	14.377	10.437	0.048	0.066	1.75	1.75	156.235	156.887	943.133	1347.664	846.3664	1426.4136
6	10.154	16.320	0.068	0.042	1.75	1.25	159.987	144.098	808.686	775.654	839.4184	856.9068
7	16.052	8.090	0.043	0.086	1.25	1.50	145.300	136.000	623.209	420.024	694.6037	460.3628
8	9.294	11.141	0.075	0.062	1.25	2.00	149.848	146.780	782.794	1180.124	822.0663	1324.3979
9	7.159	19.971	0.097	0.035	1.50	1.75	146.948	140.670	829.282	998.632	869.9433	1132.7533
10	16.153	9.720	0.043	0.071	1.50	2.00	141.287	147.987	564.362	1177.354	624.114	1248.8439
11	10.664	29.825	0.065	0.023	1.75	1.50	155.450	155.534	918.652	680.875	962.162	850.7103
12	15.612	8.736	0.044	0.079	1.50	1.75	145.560	149.243	1007.025	683.706	954.8884	771.6247
N	12	12	12	12	12	12	12	12	12	12	12	12
Mean	12.063	15.781	0.062	0.053	1.562	1.583	151.100	146.123	822.1463	1022.649	833.721158	1148.934
SD	3.4484	7.5845	0.0193	0.0214	0.1884	0.2683	6.2975	6.5965	183.9808	368.2999	98.8044198	390.0537
Minimum	7.159	8.090	0.043	0.023	1.250	1.250	141.287	136.000	564.362	420.024	369.943	460.363
Median	12.478	12.626	0.057	0.056	1.500	1.500	150.207	145.901	793.785	983.413	659.359	1177.394
Maximum	16.153	29.825	0.097	0.086	1.750	2.000	160.204	156.887	1007.025	1685.781	904.678	1815.306
CV%	28.59	48.06	30.98	40.47	12.06	16.94	4.17	4.51	22.37811	36.01	11.85	33.95
Geometric Mean	11.585	14.318	0.060	0.048	1.552	1.563	150.979	145.986	770.027	957.912	625.674	1081.677

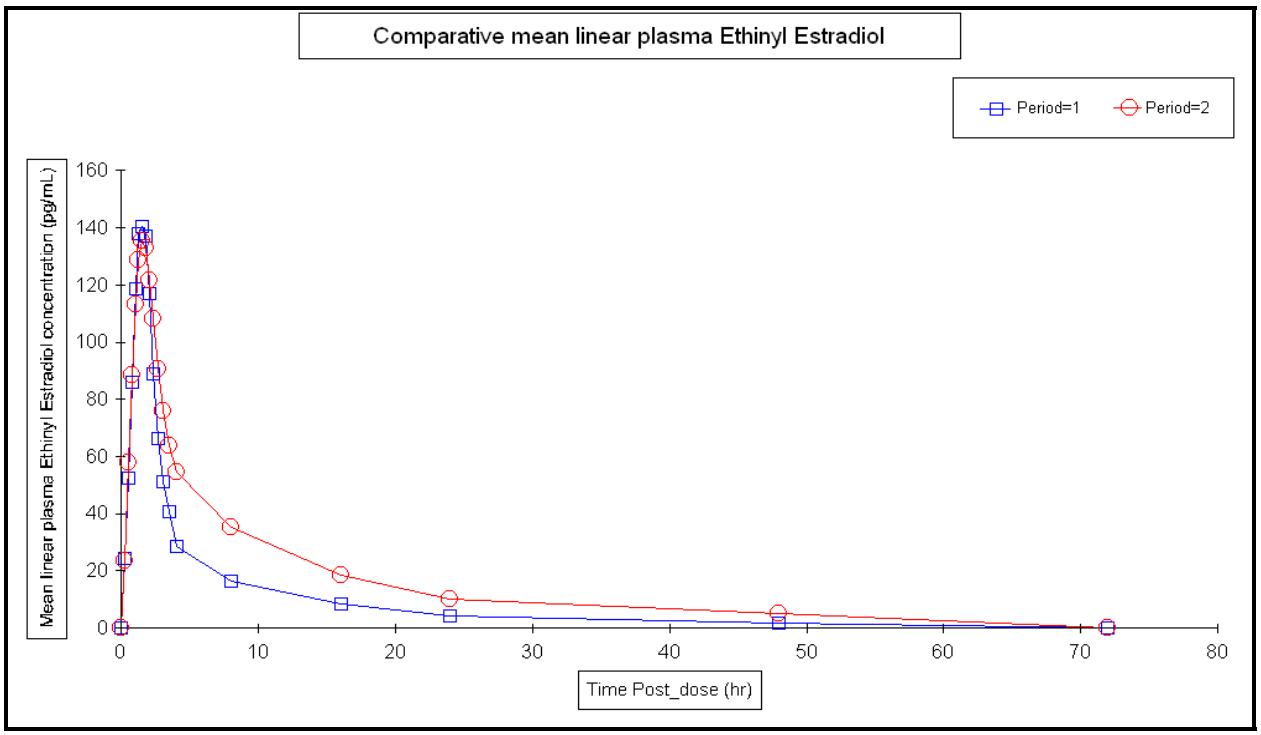

Figure No.33. Mean linear plot of plasma concentration of Ethinylestradiol (time v/s mean plasma conc.)

8.3 Pharmacokinetic parameters evaluation

Estimation and calculation of pharmacokinetic parameters was performed using the Winnonlin software version 5.0.1. The maximum Ethinyl estradiol and Levonorgestrel concentration (C_{max}) and the corresponding peak time (t_{max}) were determined by the inspection of the individual drug plasma concentration-time profiles. The elimination rate constant (K_{el}) was obtained from the least square fitted terminal log-linear portion of the plasma concentration-time profile The elimination half-life ($t_{1/2}$) was calculated as $0.693/K_{el}$. The area under the curve to the last measurable concentration (AUC_{0-t}) was calculated by the linear trapezoidal rule. The area under the curve extrapolated to infinity ($AUC_{0-\infty}$) was calculated by equation $AUC_{0-t} + C_t/K_{el}$ where C_t is the last measurable concentration.

Pharmacokinetic parameters of Levonorgestrel were compared between morning dosing and evening dosing. From the results it was revealed that the C_{max} was the same with day time and night time dosing (5.617 ng/ml and 5.646 ng/mL) dosing. Slight variation in half-life ($t_{1/2}$)

was observed (25.209 to 30.680 hr); time to reach the maximum concentration (t_{max}) was found to be faster during night time(1.771 hr) when compared to day time (2.195hr) dosing. However the % CV was found to be 27.90% and 23.25% with the subjects for morning dosing compared to evening dosing. Area under curve (AUC_{inf}) was found to be more during the night time dosing (152.261h/mL) when compared to day time dosing (138.047ng.h/mL). The elimination rate constant was more during day time when compared to the night time dosing and the results are insignificant.

Pharmacokinetic parameters of Ethinyl estradiol were compared between morning dosing and evening dosing. From the results it was revealed that the C_{max} time dosing was same with night time (151.100 ng/ml and 146.123 ng/mL) dosing and the values are clinically insignificant. Slight variations in half-life ($t_{1/2}$) were observed (12.063 to 15.781 hr) between day and night time dosing with the %CV of 28.59% and 48.06% respectively. There was no significant difference in the time to reach the maximum concentration (t_{max}) and between day (1.562 hr) and night time (2.583hr) dosing. Area under curve (AUC_{inf}) was found to be more during the night time dosing (1148.934h/mL) than day time dosing (833.047ng.h/mL).

Many physiological factors are involved in different steps of the fate of the drugs in the organism that vary along the 24 hr cycle including changes in gastrointestinal. Cardiovascular, hepatic and renal functions.

Levonorgestrel metabolism occurs by the reduction of the Δ4-3-oxo group and hydroxylation at positions 2α, 1β, and 16β, followed by sulphation and glucurodination. Metabolic clearance rates may differ among individuals by several-fold, and this may account in part for the wide variation observed in Levonorgestrel concentrations among users [87].

Ethinyl estradiol is metabolized by Cytochrome P450 enzymes (CYP3A4) in the liver by the major oxidative reaction resulting in 2-hydroxylation product. Levels of Cytochrome P450 (CYP3A) vary widely among individuals and can explain the variation in rates of ethinyl estradiol 2-hydroxylation [75].

Changes in the pharmacokinetic parameters of Levonorgestrel and ethinyl estradiol are attributed to factors like absorption, distribution, metabolism and elimination of the implicated to circadian rhythms. The factors that influence the changes in drug absorption are

circadian variation in gastric acid secretion and pH, motility, gastric emptying time, and gastrointestinal blood flow vary along the 24 hr scale[26].Some of the indirect reasons supporting this are posture and feeding conditions. Since Levonorgestrel 100% bound to the plasma proteins circadian variation in plasma proteins brings about the changes in its distribution. Since drug metabolism depends on the activities of the metabolizing enzymes and blood flow, both of these were shown to vary with 24hr cycle. This may result in variation in clearance of the drugs. Many of the renal functions like glomerular filtration, renal blood flow, urinary pH show variation in 24hr cycle with higher values during the day time. In the present study very small population was taken so the results have shown intra subject variability. The study was conducted by dosing the subjects with single dose; therefore the effect of circadian rhythm on the pharmacokinetic parameters following multiple dosing with large number of population needs to be studied in order to reduce intra-subject variability.

Chapter-9

SUMMARY

Safety of the patients is a major concern for the pharmacists and medical practitioners. The benefit/risk ratio is a prime indicator of a prescription these days. This has led to a reduction in the prescribed doses and the use of multidrug formulations replacing the single drug formulations. Resultantly, a need a need has arised for bioanalytical methods sensitive enough to detect drug levels in the range of ng/mL to pg/mL. Also a multidrug formulations demand selectivity in the bioanalytical methods, doing away with the prior separation of the analytes common to chromatographic methods. All these factors cumulatively have resulted in the popularity of LC-MS/MS technique.

A simple, rapid and rugged LC–MS/MS method was developed and validated for the determination of Budesonide, Fluticasone propionate, Prednisolone, Prednisone, Dexamethasone and Triamcinolone acetonide in human K_2EDTA plasma. The plasma volume used for the method was only 0. 500mL. The method was shown to be precise and accurate over the linearity ranges of 10.000-2000.000 pg/mL for Budesonide, 10.000 to 1000.000 pg/mL for Fluticasone propionate, 1.000 to 1000.000ng/mL for Prednisolone, 0.500 to 500.000ng/mL for Prednisone, 0.250 to 250.000ng/mL for Dexamethasone and 0.100 to 1000.000ng/mL Triamcinolone acetonide. The coefficient of determination (r^2) was found to be $\geq$ 0.9943 for all the analytes. Stability of the analytes was proved at all stages of the analysis, anticipated during clinical study. The extensive study of stability of the analytes reported in this method has not been observed in any of the methods found in literature. The method was successfully applied for the determination of Prednisolone and Prednisone in plasma samples obtained from the chronopharmacokinetic study.

A rapid, sensitive and highly selective method for the determination of Levonorgestrel in K_2EDTA plasma was developed, using LC-MS/MS. Electrospray ionization (ESI) in positive mode was used for the quantification of the Levonorgestrel. The lowest concentration quantified was 0.100 ng/mL from 0.300mL plasma sample. The coefficient of determination (r^2) was found to be $\geq$0.9963. The method was found to be linear over a concentration range of 0.100-200.000ng/mL. The calibration range was constructed by considering Cmax of the different dosage strengths of Levonorgestrel available from literature. The overall

chromatographic run time was 1.60 minutes so the method was proven to be excellent for rapid quantification of Levonorgestrel in human plasma. All the validation parameters were found to be within the acceptable limits. The method was successfully applied for the determination of Levonorgestrel in study samples obtained from the chronopharmacokinetic study.

A highly selective and sensitive method for the determination of ethinyl estradiol in human plasma was developed using liquid–liquid extraction and LC-ESI/MS/MS for detection. The sensitivity of the method for ethinyl estradiol was increased by the use of chemical derivatization with dansyl chloride. This resulted in achieving the lower limit of quantitation of 2.000 pg/mL from 0.800 mL of the plasma sample. The method was found to be linear over a large concentration range of 2.000-500.000 pg/mL. The coefficient of determination (r^2) was found to be ≥ 0.9976.The lowest concentration quantified was 2.000pg/mL with acceptable accuracy and precision. The intra-day precision and accuracy along with inter-day precision and accuracies were found to be well within the acceptance criteria. This method was successfully applied to the clinical samples after administration of ethinyl estradiol to healthy human subjects.

Several methods have been reported for the determination of mifepristone in human plasma but these are not sensitive enough to quantify, when mifepristone is dosed at very low levels . A highly sensitive and selective method to quantify the drug was developed and validated using human K_2EDTA plasma with the short run time of 1.60 min. The method was found to be linear over a concentration range of 1.000-5000.000 ng/mL. The coefficient of determination (r^2) was found to ≥ 0.9975. The lowest concentration quantified was 1.000 ng/ml using 0.300mL plasma having satisfactory accuracy and precision. The precision and accuracy data for intra-day and inter-day samples were well within the acceptance criteria. This method can be extensively applied to the clinical studies which involves different dosage strengths due to the wide range of linearity.

Chronopharmacokinetic study is used to determine time-dependent and predictable (rhythmic) changes in pharmacokinetic parameters of a drug, like maximum concentration (Cmax), time

to reach Cmax (tmax), area under the concentration time curve (AUC), and half-life ($t_{1/2}$). The pharmacokinetic parameters are also affected by the circadian changes in absorption, distribution, metabolism and excretion.

The factors influencing the pharmacokinetic process, if controlled can help to maximum benefit/ risk ratio of the drugs administered. Various literatures have been shown that the time of day is an additional variable to influence the kinetics of a drug. Neglecting the possible influence of biological rhythms on drug kinetics contributes to enhance variability in drug effect. Chronopharmacological studies are necessary to overcome such variations in the kinetic study. The time related changes in the drug effects depend upon the endogenous circadian rhythms, which include metabolic pathways. The methods developed and validated in the present study were successfully applied to the chronopharmacological studies of Prednisolone, Levonorgestrel and Ethinyl estradiol. The results for C_{max} and t_{max} were not of clinically insignificant. Only AUC_{inf}, showed slight different between morning dosing and evening dosing of Prednisolone. Levonorgestrel has shown no difference in Cmax but there was time delay in achieving the C_{max} and AUC_{inf} was slightly higher in night time dosing when compared to day time dosing. With ethinyl estradiol, there was no significant differences in C_{max} and t_{max} but the AUC_{inf} was higher in night time dosing. Since the study was done by administering single dose of low strength in very small size sample population inter-subject variability was extensively observed. Hence the study needs to be conducted in large number of sample population. The chronopharmacokinetic study at enzymatic and functional circadian rhythms at the sub cellular and molecular level needs to be studied for the better understanding of the circadian effects.

Chapter-10

PRESENTATIONS AND PUBLICATIONS

1. Chandramohan, A.K Gajjar and A.H. Khan."Novel bioanalytical method for the determination of budesonide in human plasma by liquid chromatography-tandem mass spectrometry". Deccan Pharma Journals, Volume 01, Issue 01, 29-46 (2010).

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3. 'Novel Bioanalytical Method For The Determination Of Budesonide In Human Plasma By Liquid Chromatography-Tandem Mass Spectrometry" 61st Indian Pharmaceutical Congress-2009 held in Ahmedabad (Presentation)

4. "A highly selective and sensitive method for the determination of ethinyl estradiol in human plasma by high performance liquid chromatography with tandem mass spectrometry".(Communicated)

Chapter-11

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Chapter-12

APPENDICES

Communication of decision of the University Ethics Committee

UEC/18/2008

Protocol Title	**An open label two treatment, two period, single dose bioavailability of Prednisolone(commercial available) 20mg Tablet, in 12 healthy male volunteers , under fed condition.**
Principal Investigator	**Chandramohan,**
Name and address of Institution	**Chandramohan, Scientist III, Manipal AcuNova . Manipal**
New Review	**Yes**
Date of review **Date of previous review, if revised application**	**12.06.2008**
Decision of UEC ➢ **Approved** ➢ **Approved with suggestion** ➢ **Revision** ➢ **Rejected**	**Approved**
Suggestions/ Reasons/Remarks	
Recommended for a period of	**One year**

- **This permission is only for period mentioned above. Six monthly and final**
- **Inform UEC in case of any amendments to the protocol, change of study procedure, site and investigator and premature termination of study with reasons along with summary.**
- **Inform UEC report to be submitted to UEC immediately in case of any adverse events and serious adverse events**
- **Members of UEC have right to monitor the trial with prior intimation**
- **Hand over one copy of the consent form duly signed to the person giving the consent.**

Member Secretary,
University Ethics Committee

Date: 13.06.2008

MANIPAL UNIVERSITY ETHICS COMMITTEE MANIPAL-576104

MANIPAL
UNIVERSITY
Declared as Deemed-to-be-University under Section 3 of the UGC Act, 1956

Communication of decision of the University Ethics Committee
UEC/53/2010

Protocol Title	**A open label, two period, single dose, Chronopharmacokinetic study of commercially available Ethinyl Estradiol/ Levonorgestrel tablets in 12 healthy human adult female subjects under fasting conditions.**
Principal Investigator	**Chandramohan,**
Name and address of Institution	**Chandramohan, Scientist-III,Manipal AcuNova Limited.**
New Review	**Yes**
Date of review Date of previous review, if revised application	**26.03.2010**
Decision of UEC ➢ **Approved** ➢ **Approved with suggestion** ➢ **Revision** ➢ **Rejected**	**Approved**
Suggestions/ Reasons/Remarks	
Recommended for a period of	**One year**

- **This permission is only for the period mentioned above.**
- **Inform UEC in case of any amendments to the protocol, change of study procedure, site and investigator and premature termination of study with reasons along with summary.**
- **Inform UEC immediately in case of any adverse events and serious adverse events.**
- **Submit six monthly and final reports to UEC.**
- **Members of UEC have right to monitor the trial with prior intimation.**
- **Hand over one copy of the consent form duly signed to the person giving the consent.**

Member Secretary,
University Ethics Committee

MANIPAL UNIVERSITY ETHICS COMMITTEE MANIPAL-576104

Date: 30.03.2010

MANIPAL UNIVERSITY
ETHICS COMMITTEE
MANIPAL - 576 104

STUDY PROTOCOL

AN OPEN LABEL, TWO PERIOD, SINGLE DOSE, BIO AVAILABILITY STUDY OF PREDNISOLONE 20 MG TABLETS, IN 12 HEALTHY HUMAN ADULT MALE VOLUNTEERS, UNDER FED CONDITIONS FOR CHRONOPHARMACOLOGIC EVALUATION.

STUDY CENTRE

Manipal AcuNova KH Clinical Research Centre
IV floor, Shirdi Saibaba Cancer Hospital
Manipal – 576 104, India

MANIPAL UNIVERSITY ETHICS COMMITTEE MANIPAL-576104

1 PROTOCOL SUMMARY

Study Drug:	Prednisolone(Wysolone) 20mg tablets
Title of Study:	An open label, two period, single dose, bio availability study of prednisolone (commercially available) 20 mg tablets, in 12 healthy human adult male volunteers, under fed conditions for chronopharmacologic evaluation.
Study Centre:	Manipal AcuNova KH Clinical Research Centre IV floor, Shirdi Saibaba Cancer Hospital Manipal – 576 104, India
Objectives:	• To compare the bioavailability of a single-dose oral Prednisolone (Wysolone) 20 Mg tablet at different timings of dosing in healthy human adult male volunteers under fed conditions. • To investigate the correlation between the biochemical parameters at different time of dosing in healthy human adult male volunteers under fed conditions
Screening Procedures:	Demographic data, medical history, general physical examination, ECG, chest X-ray, haematology, biochemistry, serology, urine analysis and urine screening for drug of abuse.
Housing:	All volunteers will be housed in the clinical study facility at least 10 hours before administration of the dose and will continue to remain in the clinical study facility for at least 16 hours after administration of the study medication in each study period.
Drug Administration:	One prednisolone (commercially available) 20mg tablet will be administered orally with about 240 mL of water at room temperature in each study. This activity will be followed by mouth check to assess the compliance to dosing.
Number of Volunteers:	12 healthy human adult male volunteers.
Blood Sampling:	A total of 16 (1 X 4 mL) blood samples will be collected in each of the two study periods. The pre-dose sample (1 x 4mL) will be collected 1 hour before dosing. The post-dose samples (1x 4mL) will be collected at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.50, 6.0, 8.0, 12.0 and 16.0 hour after dosing in Vacutainers containing EDTA. Pre dose sample, 1.0, 2.0, 2.5, 3.0, 6.0, 12.0 and16.0 hour samples are also collected in plain vacutainers (1X 4mL) for the estimation of biochemical parameters. Heparin-lock technique will be used to prevent clotting of blood in the indwelling cannula. Before each blood sample is drawn, 0.5 mL of blood will be discarded so as to prevent the saline diluted blood and heparin from interfering with the analysis.
Volunteer Monitoring:	Blood pressure, pulse rate, respiratory rate and oral temperature will be measured after check in, prior to dosing and at 2:00, 4:00, 6:00, 12:00 and 16:00 hours post dose in each study period.
Washout Period:	At least 7 days of washout period will be maintained between the two study periods.
Pharmacokinetic Parameter	C_{max}, T_{max}, $t_{1/2}$, AUC_{0-t}, $AUC_{0-\infty}$ and Kel of both periods will be generated using appropriate software.
Analytical Method:	The plasma concentration of prednisolone and prednisone will be analyzed using a validated Liquid Chromatography Mass Spectrometry (LC-MS/MS) method.

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2 BACKGROUND INFORMATION

2.1. Brief Introduction

a) Pharmacology of Prednisolone:

Prednisolone is a synthetic adrenocortical steroid drug with predominantly glucocorticoid properties. Some of these properties reproduce the physiological actions of endogenous glucocorticosteroids, but others do not necessarily reflect any of the adrenal hormones normal functions; they are seen only after administration of large therapeutic doses of the drug. The pharmacological effects of prednisolone which are due to its glucocorticoid properties include: promotion of gluconeogenesis; increased deposition of glycogen in the liver; inhibition of the utilization of glucose; anti-insulin activity; increased catabolism of protein; increased lipolysis; stimulation of fat synthesis and storage; increased glomerular filtration rate and resulting increase in urinary excretion of urate (creatinine excretion remains unchanged); and increased calcium excretion.

b) Pharmacokinetics of Prednisolone

Prednisolone is readily absorbed from gastro-intestinal tract. Peak plasma concentration is obtained 1-2 hours after oral administration and it had a plasma half-life of 2-3 hours. The peak concentrations after Enteric coated tablets 249 ng/ml attained at 5.14 h.

It is excreted in the urine as free and conjugated metabolites together with an appreciable proportion of unchanged prednisolone.

In humans, the pharmacokinetics of prednisolone showed diurnal variation, which was related to the concentrations of endogenous hydrocortisone. Mean peak plasma concentrations of 0.466 µg/ml prednisolone were obtained around 1 to 2 hours after oral administration of 40 mg prednisolone in the morning. Oral bioavailability was dose-dependent and showed considerable individual variations; bioavailability ranged from 60% to 92% for a 10 mg tablet.

Prednisolone was bound to plasma proteins, mostly to the corticosteroid binding globulin, transcortin, and a smaller percentage to albumin; the extent of the binding was concentration dependent.

Prednisolone competed with endogenous hydrocortisone for the binding site.

Prednisolone was widely distributed to the tissues and crossed the placenta. Following oral or Parentral dosing with 14~-prednisolone more than 90% of the administered dose was excreted in urine within 48 hours of treatment; only 1 to 2% of the dose was recovered from faeces

c) Chronopharmocology.

The assumption of homeostasis implies that the pharmacokinetics and pharmacodynamics of medications are comparable regardless of the time of day, day of menstrual cycle, and month or year of use. However, facts dispute the validity of this assumption; the time with reference to circadian rhythms of drug ingestion, injection, or infusion can affect drug behavior, sometimes markedly. Chronopharmocology is the study of the manner and extent to which medications are directly affected by biological rhythms, as opposed to other known influences, such as body posture and meal timing/content, when dosed at different times (e.g., day of week, stage of menstrual cycle, and season of year). Chronokinetics refers to dosing-time (i.e., rhythm-dependent) differences in the absorption, distribution, metabolism, and elimination of medications. Circadian rhythms in gastrointestinal pH can affect drug dissolution, and circadian rhythms in gastric emptying, motility, and blood flow can affect the rate and amount of drug absorption. Moreover, circadian rhythms in hepatic blood flow and enzyme activity can significantly affect drug biotransformation and metabolism, and rhythms in hepatic bile function and flow as well as renal blood flow, glomerular filtration, and tubular function can affect drug elimination. The pharmacokinetics of a large number of medications is influenced by circadian rhythms. Chronodynamics refers to dosing-time (i.e., rhythm-dependent) differences in the effects

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INVESTIGATORS NAME, DESIGNATION AND DECLARATION

We, the undersigned declare that we have read and understood this protocol and hereby agree to conduct the study in accordance with this protocol and to comply with all requirements of the ICH guidelines for Good Clinical Practice. We further agree to ensure that all associates assisting in the conduct of study are informed regarding their obligations.

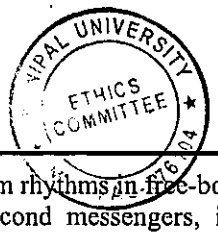
1. **Dr. Padma G. M. Rao-** Study Director
   Director BA/BE
   Manipal Acunova ltd,
   4th floor, Shirdi Saibaba cancer hospital, Manipal

2. **Dr. Vadiraja BM -**Principal Investigator
   Associate Professor
   Dept. of Radiotherapy and Oncology
   Shirdi Saibaba cancer hospital, Manipal

3. **Dr. Priya P Pillai-** Clinical Investigator
   Manipal Acunova ltd,
   4th floor, Shirdi Saibaba cancer hospital, Manipal

4. **Mr. Chandramohan-**Sub Investigator
   Scientist-III- Bioanalytical department
   Manipal Acunova ltd,
   4th floor, Shirdi Saibaba cancer hospital, Manipal

5. **Dr. Anuradha K Gajjar-** Guide
   Institute of Pharmacy, Nirma University, Ahmedabad, India



of medications, including drug dose-response. It results from rhythms in free-bound drug fraction, number and conformation of drug-specific receptors, second messengers, ion channels, rate limiting step(s) in metabolic pathways, and drug pharmacokinetics. Both the desired/beneficial and undesired/adverse effects of medications can vary with the time of treatment.

Glucocorticoids are adrenocortical steroids, both naturally occurring and synthetic, which are readily absorbed from the gastrointestinal tract. Prednisolone is a white crystalline powder, very slightly soluble in water. The chemical name for prednisone is pregna-1,4-diene-3,11,20-trione, 17,21-dihydroxy- and its molecular weight is 358.43.

2.2. Indications

Endocrine disorders, Rheumatic disorders, Collagen diseases, Dermatologic diseases, Allergic states, Ophthalmic diseases, Respiratory diseases, Hematologic disorders, Neoplastic diseases, Edematous states, Gastrointestinal diseases, Nervous system disorders

2.3. Dosage and Administration

The initial dosage of Prednisolone may vary from 5 mg to 60 mg per day depending on the specific disease entity being treated. In situations of less severity, lower doses will generally suffice, while in selected patients higher initial doses may be required.

2.4. Side Effects

Fluid and Electrolyte Disturbances :Sodium retention, Fluid retention, Congestive heart failure in susceptible patients,Potassium loss,Hypokalemic alkalosis,Hypertension
Musculoskeletal: Muscle weakness,Steroid myopathy, Loss of muscle mass, Osteoporosis, Vertebral compression fractures,Aseptic necrosis of femoral and humeral heads,Pathologic fracture of long bones
Gastrointestinal: Peptic ulcer with possible perforation and hemorrhage, Pancreatitis, Abdominal distention,Ulcerative esophagitis,
Dermatologic :Impaired wound healing,Thin fragile skin,Petechiae and ecchymoses Facial erythema,Increased sweating,May suppress reactions to skin tests,
Neurological:Convulsions,Increased intracranial pressure with papilledema (pseudotumor cerebri) usually after treatment,Vertigo, Headache
Endocrine: Menstrual irregularities,Development of Cushingoid state,Suppression of growth in children,Secondary adrenocortical and pituitary unresponsiveness, particularly in times of stress, as in trauma, surgery, or illness,Decreased carbohydrate tolerance,Manifestations of latent diabetes mellitus,Increased requirements for insulin or oral hypoglycemic agents in diabetics

Ophthalmic: Posterior subcapsular cataracts,Increased intraocular pressure, Glaucoma Exophthalmos

Metabolic: Negative nitrogen balance due to protein catabolism

2.5. Drug Interactions

Drugs that induce hepatic enzymes such as phenobarbital, phenytoin and rifampin may increase the clearance of corticosteroids and may require increases in corticosteroid dose to achieve the desired response.

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Drugs such as troleandomycin and ketoconazole may inhibit the metabolism of corticosteroids and thus decrease their clearance.

Aspirin should be used cautiously in conjunction with corticosteroids in patients suffering from hypoprothrombinemia.

2.6. Precautions

Corticosteroids should be used cautiously in patients with ocular herpes simplex because of possible corneal perforation.

The lowest possible dose of corticosteroid should be used to control the condition under treatment, and when reduction in dosage is possible, the reduction should be gradual.

Psychic derangements may appear when corticosteroids are used, ranging from euphoria, insomnia, mood swings, personality changes, and severe depression, to frank psychotic manifestations. Also, existing emotional instability or psychotic tendencies may be aggravated by corticosteroids. Aspirin should be used cautiously in conjunction with corticosteroids in hypoprothrombinemia.

Convulsions have been reported with concurrent use of methylprednisolone and cyclosporin. Since concurrent use of these agents results in a mutual inhibition of metabolism, it is possible that adverse events associated with the individual use of either drug may be more apt to occur.

2.7. Contraindications

Systemic fungal infections and known hypersensitivity to components.

3 STUDY OBJECTIVES AND PURPOSE

- To compare the bioavailability of a single-dose oral Prednisolone 20 Mg (Wysolone) at different timings of dosing in healthy human adult male volunteers under fed conditions.
- To investigate the correlation between the biochemical parameters at different time of dosing in healthy human adult male volunteers under fed conditions

4 STUDY DESIGN

4.1. Design

An open label, two treatment, two period, two sequence, single dose, bioavailability study of Prednisolone (Wysolone) 20 mg tablets in 12 healthy human adult male volunteers under fed conditions.

4.2. Number of Volunteers

12 Healthy human adult male volunteers will be included in the study.

4.3. Overall Study Plan

4.4. Healthy volunteers who were screened within 15 days prior to the first dosing day will be considered as potential participants in the study. The inclusion and exclusion criteria will be applied to all volunteers as a condition of admission into the study.

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Before admission of volunteers into the clinical study facility on the pre-study day before first dosing, the volunteers will be given full details of the study. Volunteers will be required to give their consent to participate in the study by signing an informed consent form.

The test for drugs of abuse will be done followed by pre-entry vital signs examination.

There will be a supervised overnight fasting period of at least 10 hours before dosing. A baseline sample will be taken before dosing (morning) thereafter a series of blood samples will be taken over 16 hours after the dose has been given.
After the 16 hour post-dose blood sample is taken, the volunteers will be allowed to leave after checking their vital signs.
After a washout period the volunteers will need to return to the clinical study facility for the second study period. In second period volunteers will be dosed at night.

4.5. Washout Period

At least 7 days of washout period will be given between the two study periods.

4.6. Blood Sampling

A total of 16 (1 X 4 mL) blood samples will be collected in each of the two study periods. The pre-dose sample (1 x 4mL) will be collected 1 hour before dosing. The post-dose samples (1x 4mL) will be collected at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.50, 6.0, 8.0, 12.0 and 16.0 hour after dosing in Vacutainers containing EDTA. Pre dose sample, 1.0, 2.0, 2.5, 3.0, 6.0, 12.0 and16.0 hour samples are also collected in plain vacutainers (1X 4mL) for the estimation of biochemical parameters.Heparin-lock technique will be used to prevent clotting of blood in the indwelling cannula. Before each blood sample is drawn, 0.5 mL of blood will be discarded so as to prevent the saline diluted blood and heparin from interfering with the analysis.

These sampling time points are computed to capture the entire range of concentration based on the data of study medication, which shows a peak concentration (t_{max}) at approximately 1.00 to 3.00hours.

The total volume of blood drawn including the volume necessary for the screening (12mL), post study analysis (12mL) and the volume of blood discarded (0.5 mL) before each blood draw will not exceed 240 mL per volunteer for the entire study. If the indwelling cannula does not function properly or must be removed for practical reasons, direct vein punctures will be performed.

Sample preparation
All the blood samples collected in EDTA vacutainers will be centrifuged under refrigeration (8^0C to 10^0C) of collection, at 3500 rpm for 10 minutes. The plasma is separated into a pre labelled polypropylene tubes and stored at $-70^0\,C \pm 5^0\,C$.

4.7. Study Meals

The volunteers have to fast at least 10 hours. Half an hour before dosing subject are given a normal breakfast. Water will not be permitted 1 hour before and 1 hour after study medication administration, but will be allowed at all other times. After dosing, lunch,

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snacks and dinner (day of dosing), will be served at appropriate timings from the time of dosing.

4.8. Activity Level

Volunteers should be seated upright for the first 4:00 hours following study medication administration. However, in case of an adverse event, volunteers will be appropriately placed. Volunteers will be allowed to do normal routine activity avoiding strenuous physical activity during the entire housing period of the study.

5 SELECTION AND WITHDRAWAL OF VOLUNTEERS

5.1. Volunteer Selection

The study will include 12 healthy human adult male volunteers, who voluntarily have given their written informed consent to participate in the study.

5.2. Volunteer Screening

Medical history and demographic data including sex, age, body weight (kg), and height (cm), and smoking habits will be recorded within two weeks of dosing of the drug. Each volunteer will undergo a complete general physical examination and laboratory tests for haematopoietic, hepatic and renal functions listed in section 5.3. Only medically healthy volunteers with clinically acceptable laboratory profiles and ECG will be enrolled into the study.

5.3. Clinical/Diagnostic Laboratory Tests

The clinical and laboratory tests performed during screening in clinical testing lab, Manipal AcuNova KH Clinical Research Centre are tabulated below. Reference range of the following laboratory values will be attached as Appendix II

Laboratory tests performed during the screening are tabulated below

<table>
<tr><th colspan="4">Clinical / Diagnostic Laboratory Tests</th></tr>
<tr><th>Haematology</th><th colspan="2">Serum chemistry</th><th>Urine analysis</th></tr>
<tr><td rowspan="2">▪ Haemoglobin
▪ Hematocrit
▪ Total and differential leukocyte count
▪ Red blood cell count
▪ Platelet count
▪ Erythrocyte Sedimentation Rate (ESR)</td><td colspan="2">▪ Cholesterol
▪ (Random Blood Sugar) RBS
▪ Creatinine
▪ Sodium
▪ Potassium
▪ Urea</td><td rowspan="2">▪ Colour
▪ Transparency
▪ pH
▪ Specific gravity
▪ Protein
▪ Glucose
▪ Ketones
▪ Bilirubin
▪ Blood
▪ Nitrite
▪ Urobilinogen
▪ Microscopic examination</td></tr>
<tr><td colspan="2">Liver Function Tests (LFTs)
▪ Total Bilirubin
▪ Total proteins
▪ Albumin
▪ Alanine Aminotransferase (ALT)
▪ Aspartate Aminotransferase (AST)
▪ Alkaline Phosphate (ALP)</td></tr>
<tr><th colspan="2">Serology</th><th colspan="2">Tests for drugs of abuse</th></tr>
<tr><td colspan="2">▪ Human Immunodeficiency Virus (HIV) 1 and 2
▪ Hepatitis A</td><td colspan="2">▪ Alcohol
▪ Opiates
▪ Barbiturates</td></tr>
</table>

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▪ Hepatitis B ▪ Hepatitis C (Rapid Plasma Reagin) RPR	▪ Amphetamine ▪ Benzodiazepines
Chest X-ray	**Electrocardiogram (ECG)**

5.4. Inclusion Criteria

a. Normal healthy male volunteer between 18-45 years (both age inclusive) of age, who are willing to give written consent to participate in the study

b. Volunteer must weigh at least 50 kg but not more than 90 kg and be within ± 15% of ideal body weight in relation to height, according to Life Insurance Corporation of India height and weight chart for Indian men and women

c. Volunteer who has no evidence of underlying disease during screening by medical history, physical examination and laboratory investigations performed within 2 weeks prior to commencement of the study

d. Volunteer whose pre-study screening laboratory tests are within normal limits or considered by the Investigator to be of no clinical significance

e. Volunteer with test negative for drugs of abuse(detected by Dip Stick and Enzymatic Immunoassay methods), hepatitis A, B, C and who is negative or non reactive for antibodies to HIV I and II

f. Volunteer having a 12 lead ECG recording within normal limits.

g. Volunteer must be available for the entire study period and is capable of understanding and communicating with the investigators and clinical study facility staff

5.5. Exclusion Criteria

a. Volunteer who is allergic to prednisolone or any component of the formulation and to any other related drug
b. Volunteer with history or presence of cardiovascular, pulmonary, hepatic, renal, hematological, gastrointestinal, endocrine, immunologic, dermatologic, neurological or psychiatric disease
c. More specifically, volunteer with history or presence of significant:
   Alcohol dependence, alcohol abuse or drug abuse within the past one year
   - History of chronic smoking or consumption of tobacco products
   - Asthma, urticaria or other allergic type reactions after taking any medication
   - Clinically significant illness within 4 weeks before the start of the study
   - Hypersensitivity to heparin
d. Volunteer who is scheduled for surgery within 7 days after drug administration
e. Volunteer who, through completion of any study, would have donated in excess of
   - 500 mL of blood in 14 days
   - 1000 mL of blood in 90 days

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- 1250 mL of blood in 120 days
- 1500 mL of blood in 180 days
- 2000 mL of blood in 270 days
- 2500 mL of blood in 1 year

f. Volunteer who has participated in another clinical trial involving donation of blood, within the preceding 90 -days of study start

g. Volunteer who has taken prescription medication or over-the-counter products (including vitamins and products from natural origin) within 14 days prior to administration of study medication, including topical medication meant for systemic absorption

h. Volunteer who has consumed grapefruit or grapefruit juice within 14 days prior to administration of study medication

i. Volunteer who has:
   - Systolic blood pressure less than 90 mm of Hg or more than 150 mm of Hg
   - Diastolic blood pressure less than 60 mm of Hg or more than 94 mm of Hg. Minor deviations (1-3 mm Hg) at check-in may be acceptable at the discretion of the Physician/ Principal Investigator

5.6. Restrictions and Prohibitions

Volunteers will be instructed as follows:

- Not to consume tobacco containing products (smoking, tobacco chewing, gutkha etc) and xanthine containing food and beverages, (chocolates, tea, coffee or cola drinks) for at least 24 hours before dosing and during the entire study including the washout period.

- To abstain from alcohol and all kind of fruit juices (especially grape fruit) for at least 48 hours before dosing and during the entire study including the washout period.

- Not to consume any medication (including over-the-counter products), preceding the study and for the entire duration study. This restriction includes vitamins taken as nutritional supplements for non-therapeutic indication. If concomitant medication is required during the time of sample collection or during the washout period the volunteers will be treated accordingly, and a decision to continue or discontinue the volunteers will be made by the Principle Investigator, based on (a) the time the medication was administered, (b) pharmacology and pharmacokinetic interaction of concomitant medication with the study medication.

5.7. Discontinuations and Withdrawal

A. Volunteers are free to dropout from the study at any time without stating any reason

B. The investigator can withdraw a volunteer from the study if:
   a. The volunteer suffers from significant inter current illness or undergoes surgery during the course of the study
   b. The volunteer experiences adverse event, when withdrawal would be in the best interest of the volunteer
   c. The volunteer fails to comply with the requirements of the protocol
   d. It is necessary to further protect the health of the volunteer or the integrity of the study
   e. Volunteers who vomit before two times the median t_{max}

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Assessment of the vital signs may also be done, if the attending physician finds it necessary at any time during the conduct of the study. In case of any abnormality in vital signs before dosing, medical opinion will be taken as to whether to dose the volunteer or not.

8.2. Well-being

Volunteers will be asked about their well-being on the pre-study day, on the study day prior to dosing and at 2.00, 4.00, 6.00, 12.00 and 16.00 hours after administration of the drug in each study period. In addition, at all times, volunteers may report side effects spontaneously to the monitoring staff.

8.3. Adverse Event(s)

An Adverse Event (AE) is any untoward medical occurrence in a patient or clinical investigation volunteer administered a pharmaceutical product, which does not necessarily have a causal relationship with this treatment. An AE can therefore be any unfavourable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporarily associated with the use of a medicinal (investigational) product, whether or not related to the medicinal (investigational) product.

Serious Adverse Event (SAE) or Serious Adverse Drug Reaction (Serious ADR):

Any untoward medical occurrence that at any dose:

- Result in death
- Is life-threatening
- Requires inpatient hospitalisation or prolongation of existing hospitalisation
- Results in persistent or significant disability/incapacity or
- Is a congenital anomaly / birth defect

Adverse events may be classified as:

- **Mild**: causing no limitation of usual activity although the patient may experience slight discomfort.
- **Moderate**: causing some, limitation of usual activities and the patient may experience annoying discomfort.
- **Severe:** causing inability to carry out usual activities and the patient may experience intolerable discomfort or pain.

Volunteers will be monitored for adverse events during the study periods. The study physician or a medically qualified designate will be on site during dosing and for at least 6 hours post dosing. Thereafter he / she will be on call throughout the study period.

All adverse events and treatment administered will be recorded in the final report of the study.

Any volunteer who develops any adverse event or a laboratory test value outside the normal range and clinically significant will be evaluated by the clinical Investigator. The volunteer will be treated and/or followed up until the symptoms or values return to normal or acceptable levels, as judged by the Clinical Investigator. A Physician, either at the study

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6 TREATMENT OF VOLUNTEERS

6.1. Housing

All volunteers will be housed in the clinical study facility at least 10 hours before administration of the study medication, and will continue to remain in the clinical study facility for at least 16 hours after administration in each study period.

6.2. Dosing

After a normal breakfast, a single dose of prednisone 20mg tablet will be administered orally to the volunteer with about 240 mL of water at room temperature.

7 ASSESSMENT OF BIOAVAILABILITY

Pharmacokinetic analysis will include data from first 12 volunteers who successfully completes the study. In case of volunteers withdrawn or dropping out of the study, samples will not be analyzed.

Pharmacokinetic parameters for prednisolone will be calculated using appropriate software as follows:

C_{max}: Maximum measured plasma concentration during the time span specified

t_{max}: Time to the maximum measured plasma concentration. If the maximum value occurs at more than one time point, T_{max} is defined as the first time point of the maximum measured plasma concentration.

AUC_{0-t}: The area under the plasma concentration versus time curve, from time 0 to the last measurable concentration, where t = time of last identifiable concentration.

$AUC_{0-\infty}$: The area under the plasma concentration versus time curve from time 0 to infinity.

$t_{½}$: The terminal elimination half-life derived from the slope (Kel) of the elimination phase and calculated as 0.693/Kel

Kel: The elimination rate constant

8 ASSESSMENT OF SAFETY

8.1. Vital Signs

The supervising physician/clinical investigator or nurse will measure the following vital signs: oral temperature, supine blood pressure (both sitting and supine if the reading of blood pressure is outside normal range), pulse rate and respiration. Such examination will be done on the pre-study day, on the study day prior to dosing and at 2.00, 4.00, 6.00, 12.00 and 16:00 after administration of drug.

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centre of Manipal AcuNova Ltd or at a nearby hospital, will administer treatment for any serious adverse reactions.

All serious adverse events will be informed to the chairman of independent ethics committee within 24 hrs and a written document with details of the adverse event. At the beginning of the second period, volunteers will be questioned concerning unusual symptoms, which may have occurred after the previous administration of the study drug. In addition, volunteers will be regularly questioned about their well being throughout the course of their stay in the study centre of the Manipal AcuNova Ltd.

9 ANALYTICAL PROCEDURES

9.1. Analytical Method

Plasma concentrations of prednisolone will be analyzed using a validated LC-MS/MS method. The plasma concentration of the first 12 volunteers who complete the study will be analyzed.

10 STATISTICS

Statistical analyses will be performed on plasma concentration values.

11 ETHICS

12.1 Basic Principles

This research will be carried out in accordance with the principles of Declaration of Helsinki(Ethical Principles for Medical Research Involving Human Volunteers, revised by the WMA General Assembly, Tokyo, 2004), 'ICH GCP', National Regulations (ICMR Guidelines), European Guidelines, 'Indian GCP', and 'Schedule Y' of Indian Drugs and Cosmetics Act.

12.2 Institutional Ethics Committee

The Institutional Ethics Committee will review this protocol. The study will not start until the committee has approved the protocol or a modification thereof.

12.3 Informed Consent

Screening will be done as a two stage procedure. During the first stage general screening for participation in a clinical trial will be done after a written informed consent within 15 days prior to study start.

During the second stage, the purpose of the study, the procedures to be carried out and the possible potential hazards will be described to the volunteers, in a language that the volunteer comprehends. The volunteers will be required to read and sign a consent form summarizing the discussion prior to check-in. A copy of the informed consent form will be given to the volunteer prior to check-in which describes the study procedures and the possible potential hazards in non technical

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terms in conformity with regulatory requirements. By signing the consent form, the witness attests that the information in the consent form and any other written information was accurately explained to and apparently understood by the volunteer and that the informed consent was freely given by the volunteer. The Informed Consent Form (ICF) will be signed and dated by the volunteer and the Principal Investigator / Clinical Investigator and then the volunteer will be checked into the clinical study facility.

12.4 Termination of the Study

The Principal Investigator reserves the right to discontinue the study for safety reasons at any time.

12 VOLUNTEER SAFETY

If the volunteer suffers discomfort or injury as a result of study medication administration or any of the procedures carried out during the study, medical treatment will be given by a physician either at Manipal AcuNova Ltd or at a nearest convenient hospital and the expenses of the same will be met by Sub Investigator.

13 PUBLICATION POLICY

The publication policy will be at the discretion of the Sub investigator. If published the volunteers' identity will not be revealed.

14 REFERENCES

a. http://www.rxlist.com/cgi/generic/prednistab.htm

15 APPENDICES

Appendix I : Informed Consent Form

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INFORMED CONSENT FORM FOR ENROLLMENT

STUDY TITLE

AN OPEN LABEL, TWO PERIOD, SINGLE DOSE, BIO AVAILABILITY STUDY OF PREDNISOLONE 20 MG TABLETS, IN 12 HEALTHY HUMAN ADULT MALE VOLUNTEERS, UNDER FED CONDITIONS FOR CHRONOPHARMACOLOGIC EVALUATION

1. RATIONALE AND OBJECTIVES

- To compare the bioavailability of a single-dose oral Prednisolone (Wysolone) 20 Mg tablet at different timings of dosing in healthy human adult male volunteers under fed conditions.
- To investigate the correlation between the biochemical parameters at different time of dosing in healthy human adult male volunteers under fed conditions

2. DRUG INFORMATION

Naturally occurring glucocorticoids (hydrocortisone and cortisone), which also have salt-retaining properties, are used as replacement therapy in adrenocortical deficiency states. Prednisolone is primarily used for its potent anti-inflammatory effects in disorders of many organ systems.

Glucocorticoids cause profound and varied metabolic effects. In addition, they modify the body's immune responses to diverse stimuli.

3. STUDY PROCEDURE

There will be 12 healthy human adult male subjects participating in the study and the study will be conducted at Manipal AcuNova KH Clinical Research Center, Manipal. If selected, you will be one of the 12 subjects participating in this study.

The study consists of two periods. Each study period will be separated by a washout period of at least 7 days to ensure that the study medicine taken during first period of the study is no longer available in your body.

During each study period you will be housed in the clinical study facility at least 10.00 hours before administration of the study medicine and you will continue to stay here for at least 16.00 hours after administration of the study drug, therefore you will be housed for atleast 26 hours in Manipal AcuNova KH Clinical Research Center, Manipal.

You are required to take single dose of prednisolone(Wysolone) 20 mg, in each of the two study periods, orally. In first period you will be dosed in the morning and in the second period you will be dosed at night. Thus, at the end of the two study periods; you would have taken the two doses of Prednisolone (Wysolone) 20 mg.

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In each period, you are required to fast at least 10 hours prior to administration of the study drug. You will receive normal breakfast at least half an hour prior to receive a single dose prednisolone(commercially available) 20 mg with 240 mL of water. You will not be allowed to drink water for 1 hour before and 1 hour after study medicine administration. Thereafter, water will be allowed at all times.

Standardized lunch, snacks and dinner will be served at appropriate timings after administration of the study medication respectively during each study period. Meal plan will be identical for the two study periods.

You will leave the clinical study facility after the 16 hour blood collection (provided your vital signs are within normal limits).

Collection of Blood Samples

A total of 16 (1 X 4 mL) blood samples will be collected in each of the two study periods. The pre-dose sample (1 x 4mL) will be collected 1 hour before dosing. The post-dose samples (1x 4mL) will be collected at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.50, 6.0, 8.0, 12.0 and 16.0 hour after dosing in vacutainers containing EDTA. Pre dose sample, 1.0, 2.0, 2.5, 3.0, 6.0, 12.0 and 16.0 hour samples are also collected in plain vacutainers (1X 4mL) for the estimation of biochemical parameters in both the periods.

Heparin-lock technique (wherein 0.5 mL of flush solution of Heparin 10 USP units/mL will be injected into the indwelling cannula, after each blood draw) will be used to prevent clotting of blood in the indwelling cannula. Before each blood sample is drawn, 0.5 mL of blood will be discarded so as to prevent the heparin and saline in the cannula from interfering with the drug analysis. In case the indwelling cannula does not function properly or it must be removed for practical reasons, blood samples will be collected by direct vein puncture.

After 16 hour post-dose blood sample is taken, you will be allowed to leave the clinical study facility after having your vital signs checked and provided they are satisfactory.
After collecting 16.00 hour blood sample in period 2, (1×12 mL) of blood will be drawn for performing post study safety analysis to assess safety.

The total volume of blood drawn including the volume necessary for the laboratory tests, the volume of blood discarded before each blood draw and post study safety analysis will be 232 mL per subject for the entire study as mentioned below.

Volume of each sample: 4 mL
Total number of samples: 48 (for all the two periods)
Volume of discarded blood: 16 mL (0.5 ml discarded at each sampling time point)
Volume of blood collected during screening: 12 mL
Volume of blood collected for post-study safety analysis at the end of the study: 12 mL
Total amount of blood collected: 232 mL
Your participation in the study will be completed after the final blood draw for post-study safety analysis. During the entire study period, you are advised not to donate blood. If in case, you have to do so, inform Manipal AcuNova KH Clinical Research Center, Manipal, before donating. As a measure of comparison a standard Red Cross blood donation is 450 ml in any 10 week period.

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You are advised not to give more than 900 ml of blood in next 20 week period after completion of the study.

In order to ensure your safety and well-being during the course of the study, your vital signs (oral temperature, blood pressure, pulse rate and respiratory rate) will be recorded and you will be asked how you feel before check-in, before administration of study medicine and at around 2.00, 4.00, 6.00, 12.00 and 16:00 after administration of drug.

4. RESTRICTIONS

Volunteers will be instructed as follows:

1. Not to consume tobacco containing products (smoking, tobacco chewing, gutkha etc) and xanthine containing food and beverages, (chocolates, tea, coffee or cola drinks) for at least 24 hours before dosing and during the entire study including the washout period.

2. To abstain from alcohol and all kind of fruit juices (especially grape fruit) for at least 48 hours before dosing and during the entire study including the washout period.

3. Not to consume any medication (including over-the-counter products), preceding the study and for the entire duration study. This restriction includes vitamins taken as nutritional supplements for non-therapeutic indication. If concomitant medication is required during the time of sample collection or during the washout period the volunteers will be treated accordingly, and a decision to continue or discontinue the volunteers will be made by the Principle Investigator, based on (a) the time the medication was administered, (b) pharmacology and pharmacokinetic interaction of concomitant medication with the study medication.
4. You will also be required to maintain the posture (sitting upright) for first 4 hours after dosing.

5. RISKS, DISCOMFORTS AND PRECAUTIONS ASSOCIATED WITH PARTICIPATION IN THIS STUDY

A single large dose of prednisolone is not expected to produce life-threatening symptoms. However, long-term use of high steroid doses can lead to symptoms such as thinning skin, easy bruising, changes in the shape or location of body fat (especially in your face, neck, back, and waist), increased acne or facial hair, menstrual problems, impotence, or loss of interest in sex.

Warning: Persons who are on drugs which suppress the immune system are more susceptible to infections than healthy individuals. Chickenpox and measles, for example, can have a more serious or even fatal course in non-immune children or adults on corticosteroids. In such children or adults who have not had these diseases, particular care should be taken to avoid exposure

Withholding information concerning previous or present medical problems could be dangerous to your health.

7. BENEFITS AND VOLUNTARY NATURE OF PARTICIPATION

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Since you do not require treatment with this drug, you will receive no medical benefit from this study. You are entirely free to participate or refuse to participate in this study. Travelling expenses and for the time spent in the clinical facility each volunteer will be compensated Rs. 500/= per period.

8. CONFIDENTIALITY

The records of your medical history, physical examination, laboratory results and any other information or data generated during this study will be made available only to the ethics/research committee.

9 COMPENSATION AND MEDICAL TREATMENT FOR INJURY

If you suffer from any injury related to this study, medical treatment will be provided free of cost at nearest convenient hospital and the expenses of the same will be met by Sub Investigator.

10 CONTACTS FOR ADDITIONAL INFORMATION

You can clarify any doubts or questions related to this study physician at the centre. However if you require further information related to this study or your rights as a subject you can contact,

Dr N. Udupa, Member, University Ethics Committee, Manipal (Phone No. 2922433)

Dr. Padma G.M Rao, Director (BA/BE), Manipal AcuNova KH Clinical Research Center, Manipal (Phone No.2571201 Extn: 22553)

Dr. Vadiraj BM, Principal Investigator, Manipal AcuNova KH Clinical Research Center, Manipal (Phone No +91-9845221606)

Sign: _____

Declaration by the volunteer

1. I confirm that I have read and understood the information provided in the consent form, which details about the study and have had the opportunity to clarify my doubts.
2. I understand that my participation in the study is voluntary and that I am free to discontinue at any time, without my medical care and legal rights being affected.
3. I understand that my participation may be terminated by the Principal Investigator in the interest and safety of my health.
4. I understand that the ethics committee will not need my permission to look at my health records both in respect of the current study and any further research that may be conducted in relation to it, even if I discontinue from the study.
5. I understand that my identity will not be revealed or released to third parties or published.
6. I agree to take part in the above study.
7. I declare that I had given all the details of the consent to my family members.

Informed consent given by:

Name :

Sign _____ Date: _____ Time : _____

Address:_____

_____

Informed consent discussion conducted and queries answered by:

Sign _____ Date : _____ Time:

Name: _____

Sign: _____

MANIPAL UNIVERSITY ETHICS COMMITTEE MANIPAL-576104

TITLE

A OPEN LABEL, TWO PERIOD, SINGLE DOSE, CHRONOPHARMACOKINETIC STUDY OF COMMERCIALLY AVAILABLE ETHINYL ESTRADIOL/ LEVONORGESTREL (0.05MG /0.25MG)TABLETS IN 12 HEALTHY HUMAN ADULT FEMALE SUBJECTS UNDER FASTING CONDITIONS.

Submitted By:
Mr. Chandramohan
Scientist-III
IV floor, Sri Shirdi Sai Baba Cancer Hospital & Research Centre,
Manipal- 576 104, India

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Investigators Declaration

I accept that the sponsor and the Independent Ethics Committee must approve the protocol and subsequent changes to the protocol in writing before its implementation.

We, the undersigned declare that we have read and understood this protocol and hereby agree to conduct the study in accordance with all requirements of the current version of the Declaration of Helsinki, the current ICH Guidelines for Good Clinical Practices and Good Laboratory Practices, regulatory requirements of USFDA and relevant National Laws and Regulations. We further agree to ensure that all associates assisting in the conduct of the study are informed regarding their obligations.

We agree to comply with all relevant Standard Operating Procedures required for the conduct of this study and would document any deviation occurring during study.

24 MAR 2010

Dr. S. T. Balamurali — **Date**

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24MAR2010

Dr. Janhvi D.H. — **Date**

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24 MAR 2010

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Personal and Facilities Involved

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Protocol Summary

Study Drug:	Ethinyl estradiol and Levonorgestrel (0.05mg /0.25mg)tablet
Title of Study:	A open label, two period, single dose, Chronopharmacokinetic study of commercially available Ethinylestradiol and Levonorgestrel (0.05mg /0.25mg) tablet in 12 healthy human adult female subjects under fasting conditions.
Investigators:	Principal Investigator: Dr. S. T. Balamurali Clinical Investigator: Dr. Jahnavi D.H. Sub Investigator: Mr. Chandramohan
Clinical Study Centre:	Manipal AcuNova KH Clinical Research Centre, IV floor, Sri Shirdi Sai Baba Cancer Hospital & Research Centre, Manipal- 576 104, India.
Objectives:	The primary objective of this study is to compare the single-dose oral bioavailability of Ethinylestradiol/ Levonorgestrel in healthy human adult female subjects on different time of dosing under fasting conditions.
Screening Procedures:	Demographic data, medical history, general physical examination, 12-lead ECG, chest X-ray (PA view), hematology, clinical bio-chemistry, serology, urine analysis, urine pregnancy test, tests for alcohol and drugs of abuse.
Housing:	All subjects will be housed in the clinical study facility at least 11.00 hours before administration of the IP and will continue to remain in the clinical study facility for at least 24.00 hours after administration of the IP in each study period.
Drug Administration:	After fasting for at least 10.00 hours, one tablet of Ethinylestradiol and Levonorgestrel tablet will be administered orally to each subject in sitting posture, with 240 ± 2 mL of water at room temperature, in each study period.
Number of Subjects:	12 healthy human adult subjects
Blood Sampling:	A total of 19 (1×4.0 mL) blood samples will be collected from each subject in pre-specified vacuum tubes containing K2 EDTA in each study period. The pre-dose sample (1×4.0 mL) will be collected within 01.00 hour before dosing in each study period. The post-dose samples (1×4.0 mL) will be collected at 00.25, 00.50, 00.75, 01.00, 01.25, 01.50, 01.75, 02.00, 02.33, 02.67, 03.00, 03.50, 04.00, 08.00, 16.00,

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	Meal plan will be identical for both periods of the study. .
Washout Period:	At least 07 days of washout period will be maintained between IP administrations in each treatment period.
Pharmacokinetic Parameters:	Primary pharmacokinetic parameters: C_{max}, AUC_{0-t} and $AUC_{0-\infty}$. Secondary pharmacokinetic parameters: t_{max}, $t_{½}$, and K_{el}.
Analytical Method:	The plasma concentration will be analyzed using a validated Liquid Chromatography Mass Spectrometry (LC-MS/MS) method.

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	24.00, 48.00 and 72.00hours after dosing in each study period. All the blood samples up to 12.00 hrs will be collected from an ante-cubital vein and 24.00, 48.00 and 72.00hr samples will be collected by direct vein punctures. The indwelling cannula will be removed earlier in case it does not function properly or is blocked or must be removed for practical reasons and direct vein punctures will be performed. The heparin-lock technique will be used to prevent clotting of blood in the indwelling cannula. Before each blood sample is drawn via indwelling cannula, 0.5 mL of blood will be discarded so as to prevent the saline diluted blood and heparin from interfering with the analysis. The total volume of blood drawn including the volume necessary for the screening (12 mL), post-study safety analysis (08 mL) and the volume of blood discarded (0.5 mL) before each blood sample is drawn via indwelling cannula, will not exceed 188.00 mL per subject for the entire study.
Subject Monitoring:	Vital Signs: Vital signs (blood pressure, pulse rate, respiratory rate and oral temperature) will be measured before check-in, prior to dosing on dosing day and post dose at 02.00, 04.00, 06.00 ,12.00 hours and during the ambulatory samples or on discontinuation of subject from study in each study period. All these measurements (except check-in and pre-dose vital signs examinations) will be performed within ± 01.00 hour of the scheduled time so as not to interfere with scheduled blood sampling times or meals. Well being: Subjects will be asked about their well-being before check in, prior to dosing on dosing day and approximately at 02.00, 04.00, 06.00 and 12.00 hours and during the ambulatory samples or on discontinuation of subject from study in each study period. In addition, at all times, subjects may report side effects spontaneously to the monitoring staff. The same will also be recorded.
Study Meals:	In each study period, subjects will be provided with standardized dinner on pre-study day, thereafter subjects have to fast overnight (for at least 10.00 hours before dosing) and for 04.00 hours after dosing. Water will not be permitted 01.00 hour before and 01.00 hour after IP administration (except 240 ± 2 mL allowed at the time of dosing), but will be allowed at all other times. After dosing, standardized lunch and snacks will be served at 04.00 and 08.00 hours respectively from the time of dosing in the two study periods.

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1 BACKGROUND & INFORMATION

Brief Introduction

In this study, the commercially available Ethinylestradiol and Levonorgestrel (0.05mg /0.25mg) tablets bioavailability was compared after dosing at different timings of the day in each period in 12 healthy human adult subjects under fasting conditions.

Drug information

Introduction

Ethinyl estradiol and levonorgestrel are forms of estrogen and progesterone, which are both female hormones involved in conception.

Ethinyl estradiol and levonorgestrel are used together in this product as an emergency contraceptive (EC) to prevent pregnancy after contraceptive failure or unprotected intercourse. Ethinyl estradiol and levonorgestrel prevent ovulation (the release of an egg from an ovary), disrupt fertilization (joining of the egg and sperm), and inhibit implantation (attachment of a fertilized egg to the uterus).

Pharmacology

Mode of Action

Combination oral contraceptives act by suppression of gonadotropins. Although the primary mechanism of this action is inhibition of ovulation, other alterations include changes in the cervical mucus which increase the difficulty of sperm entry into the uterus) and the endometrium (which reduce the likelihood of implantation).

Pharmacokinetics

Absorption

No specific investigation of the absolute bioavailability of Ethinylestradiol/ Levonorgestrel in humans has been conducted. However, literature indicates that levonorgestrel is rapidly and completely absorbed after oral administration (bioavailability about 100%) and is not subject to first-pass metabolism. Ethinyl estradiol is rapidly and almost completely absorbed from the gastrointestinal tract but, due to first-pass metabolism in gut mucosa and liver, the bioavailability of ethinyl estradiol is between 38% and 48%.

After a single dose of Ethinylestradiol and Levonorgestrel (0.02mg/0.010mg) to 22 women under fasting conditions, maximum serum concentrations of levonorgestrel are 2.8 ± 0.9 ng/mL (mean ± SD) at 1.6 ± 0.9 hours. At steady state, attained from day 19 onwards, maximum levonorgestrel concentrations of 6.0 ± 2.7 ng/mL are reached at 1.5 ± 0.5 hours after the daily dose. The minimum serum levels of levonorgestrel at steady state are 1.9 ± 1.0 ng/mL. Observed levonorgestrel concentrations increased from day 1 (single dose) to days 6 and 21 (multiple doses) by 34% and 96%, respectively (Figure 1). Unbound levonorgestrel concentrations increased from day 1 to days 6 and 21 by 25% and 83%, respectively. The kinetics of total levonorgestrel are non-linear due to an

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increase in binding of levonorgestrel to sex hormone binding globulin (SHBG), which is attributed to increased SHBG levels that are induced by the daily administration of ethinyl estradiol.

Following a single dose, maximum serum concentrations of ethinyl estradiol of 62 ± 21 pg/mL are reached at 1.5 ± 0.5 hours. At steady state, attained from at least day 6 onwards, maximum concentrations of ethinyl estradiol were 77 ± 30 pg/mL and were reached at 1.3 ± 0.7 hours after the daily dose. The minimum serum levels of ethinyl estradiol at steady state are 10.5 ± 5.1 pg/mL. Ethinyl estradiol concentrations did not increase from days 1 to 6, but did increase by 19% from days 1 to 21.

Distribution

Levonorgestrel in serum is primarily bound to SHBG. Ethinyl estradiol is about 97% bound to plasma albumin. Ethinyl estradiol does not bind to SHBG, but induces SHBG synthesis.

Metabolism

Levonorgestrel: The most important metabolic pathway occurs in the reduction of the Δ4-3-oxo group and hydroxylation at positions 2α, 1β, and 16β, followed by conjugation. Most of the metabolites that circulate in the blood are sulfates of 3α,5β-tetrahydro-levonorgestrel, while excretion occurs predominantly in the form of glucuronides. Some of the parent levonorgestrel also circulates as 17β-sulfate. Metabolic clearance rates may differ among individuals by several-fold, and this may account in part for the wide variation observed in levonorgestrel concentrations among users.

Ethinyl estradiol: Cytochrome P450 enzymes (CYP3A4) in the liver are responsible for the 2-hydroxylation that is the major oxidative reaction. The 2-hydroxy metabolite is further transformed by methylation and glucuronidation prior to urinary and fecal excretion. Levels of Cytochrome P450 (CYP3A) vary widely among individuals and can explain the variation in rates of ethinyl estradiol 2-hydroxylation. Ethinyl estradiol is excreted in the urine and feces as glucuronide and sulfate conjugates, and undergoes enterohepatic circulation.

Excretion

The elimination half-life for levonorgestrel is approximately 36 ± 13 hours at steady state. Levonorgestrel and its metabolites are primarily excreted in the urine (40% to 68%) and about 16% to 48% are excreted in feces. The elimination half-life of ethinyl estradiol is 18 ± 4.7 hours at steady state.

Dosage and administration

To achieve maximum contraceptive effectiveness, levonorgestrel and ethinyl estradiol tablets must be taken exactly as directed and at intervals not exceeding 24 hours. The dosage of levonorgestrel and ethinyl estradiol tablets is one pink tablet daily for 21 consecutive days, followed by one light green inert tablet daily for 7 consecutive days, according to the prescribed schedule. It is recommended that levonorgestrel and ethinyl estradiol tablets be taken at the same time each day.

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Contraindications

Combination oral contraceptives should not be used in women with any of the following conditions:

Thrombophlebitis or thromboembolic disorders. A past history of deep-vein thrombophlebitis or thromboembolic disorders. Cerebrovascular or coronary artery disease (current or past history) Thrombogenic valvulopathies.Thrombogenic rhythm disorders.Major surgery with prolonged immobilization Diabetes with vascular involvement.Headaches with focal neurological symptoms. Uncontrolled hypertension. Known or suspected carcinoma of the breast or personal history of breast cancer. Carcinoma of the endometrium or other known or suspected estrogen-dependent neoplasia.Undiagnosed abnormal genital bleeding.Cholestatic jaundice of pregnancy or jaundice with prior pill use Hepatic adenomas or carcinomas, or active liver disease, as long as liver function has not returned to normal.Known or suspected pregnancy Hypersensitivity to any of the components of levonorgestrel and ethinyl estradiol.

Warnings

The use of oral contraceptives is associated with increased risks of several serious conditions including venous and arterial thrombotic and thromboembolic events (such as myocardial infarction, thromboembolism, and stroke), hepaticneoplasia, gallbladder disease, and hypertension, although the risk of seriousmorbidity or mortality is very small in healthy women without underlying risk factors. The risk of morbidity and mortality increases significantly in the presence of other underlying risk factors such as certain inherited or acquiredthrombophilias, hypertension, hyperlipidemias, obesity, diabetes, and surgery ortrauma with increased risk of thrombosis.

Practitioners prescribing oral contraceptives should be familiar with the following information relating to these risks.

The information contained in this package insert is principally based on studies carried out in patients who used oral contraceptives with higher doses ofestrogens and progestogens than those in common use today. The effect of long-term use of the oral contraceptives with lower doses of both estrogens and progestogens remains to be determined.

2 STUDY OBJECTIVES AND PURPOSE

Primary Objective

The primary objective of this study is to compare the single-dose oral bioavailability of commercially available Ethinylestradiol and Levonorgestrel tablets in healthy human adult female subjects on different time of dosing under fasting conditions.

Secondary Objective

The secondary objective of this study is to monitor the safety and tolerability of a single dose of Ethinylestradiol and Levonorgestrel tablets when administered in healthy human adult subjects under fasting conditions.

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3 STUDY DESIGN

Design

A open label, two period, single dose, chronopharmacokinetic study of commercially available Ethinylestradiol and Levonorgestrel tablets in 12 healthy human adult female subjects under fasting conditions.

Study Treatment Allocation			
Group	N	Period 1	Period 2
I	12	Dosed at 8.00AM	Dosed at 8.00PM

Number of Subjects

12 healthy human adult subjects will be included in the study.

Overall Study Plan

Subjects from the pool of healthy volunteers who were screened within 21 days prior to the study start will be considered as potential participants in the study. The inclusion and exclusion criteria will be applied to all volunteers as a condition of enrollment into the study.

Before enrollment of subjects into the study, the volunteers will be given full details of the study and then will be required to give their consent to participate in the study by signing an Informed Consent Form (ICF).

Period 1 Dosed at 8.00AM	
Day 0 (Pre-Study day)	Urine pregnancy test test for alcohol and drugs of abuse, Informed Consent Process, volunteer history, vital signs examination, enquiry about well-being, application of inclusion-exclusion criteria followed by enrollment of volunteers into the study and check-in of the subjects into clinical study facility, catering, and start of 10.00 hours overnight fasting.
Day 1 (Dosing day)	Cannulation, pre-dose blood sampling, vital signs examination, enquiry about well-being, fitness check, allocated dose of IP given at scheduled time and continued fasting for next 04.00 hours. Blood sample collection, vital signs examination, enquiry of well-being, catering of study meals at scheduled times and checkout, provided that the 24.00 hours vital signs examination and enquiry of well-being is satisfactory.
Ambulatory samples	36.00, 48.00, 72.00 and 120.00hrs after dosing
Washout period of at least 07 days calculated from the day of dosing.	

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Period 2 Dosed at 8.00 PM	
Day 0 (Pre-Study day)	Urine pregnancy test, test for alcohol and drugs of abuse, volunteer history, vital signs examination, enquiry about well-being, check-in of the subjects into clinical study facility, catering, and start of 10.00 hours fasting.
Day 1 (Dosing day)	Cannulation, pre-dose blood sampling, vital signs examination, enquiry about well-being, fitness check, allocated dose of IP given at scheduled time and continued fasting for next 04.00 hours. Blood sample collection, vital signs examination, enquiry of well-being, catering of study meals at scheduled times and checkout, provided that the 24.00 hours vital signs examination and enquiry of well-being is satisfactory
Ambulatory samples	36.00, 48.00, 72.00 and 120.00hrs after dosing
Safety samples	After 12.00 hours blood sampling, a blood sample (2×4 mL) will also be collected for performing post-study safety analysis to assess safety of study subjects. This includes hematology, clinical bio- chemistry and urine pregnancy test.

Housing

All subjects will be housed in the clinical study facility at least 11.00 hours before administration of the IP and will continue to remain in the clinical study facility for at least 12.00 hours after administration drug in each study period.

Wash-out Period

At least 14 days of washout period will be maintained between investigational product administrations in the two study periods.

Blood Sampling

A total of 19 (1×4.0 mL) blood samples will be collected from each subject in pre-specified vacuum tubes containing K2 EDTA in each study period. The pre-dose sample (1×4.0 mL) will be collected within 01.00 hour before dosing in each study period. The post-dose samples (1×4.0 mL) will be collected at 00.25, 00.50, 00.75, 01.00, 01.25, 01.50, 01.75, 02.00, 02.33, 02.67, 03.00, 03.50, 04.00, 08.00, 16.00, 24.00, 48.00 and 72.00hours after dosing in each study period.

All the blood samples up to 16.00 hrs will be collected from an ante-cubital vein and 24.00, 48.00 and 72.00hr samples will be collected by direct vein punctures.

The indwelling cannula will be removed earlier in case it does not function properly or is blocked or must be removed for practical reasons and direct vein punctures will be performed.

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The heparin-lock technique will be used to prevent clotting of blood in the indwelling cannula. Before each blood sample is drawn via indwelling cannula, 0.5 mL of blood will be discarded so as to prevent the saline diluted blood and heparin from interfering with the analysis.

The total volume of blood drawn including the volume necessary for the screening (12 mL), post-study safety analysis (08 mL) and the volume of blood discarded (0.5 mL) before each blood sample is drawn via indwelling cannula, will not exceed 188.00 mL per subject for the entire study.

Extra blood sample may be collected for repeat laboratory test(s), if required, at the discretion of the investigator

Sample Separation

After collection of blood sample(s), all the blood samples will be transferred in a box with pre-cooled gel refrigerant packs, and centrifuged at 3500 rpm under refrigeration at 4° C for 5 min. After centrifugation, the separated plasma will be transferred into suitably labeled polypropylene tubes. All plasma samples will be stored upright in a deep freezer set at –70°C ± 5°C.

Study Meals

In Period I, all the subjects will be provided with standardized dinner on pre-study day, thereafter subjects have to fast overnight (for at least 10.00 hours before dosing) and for 04.00 hours after dosing. Water will not be permitted 01.00 hour before and 01.00 hour after drug administration (except 240 ± 2 mL allowed at the time of dosing), but will be allowed at all other times.

In Period II, all the subjects will be provided with standardized meal in morning on study day, thereafter subjects have to fast (for at least 10.00 hours before dosing) and for 04.00 hours after dosing. Water will not be permitted 01.00 hour before and 01.00 hour after drug administration (except 240 ± 2 mL allowed at the time of dosing), but will be allowed at all other times.

After dosing, standardized lunch and snacks will be provided at 04.00 and 08.00 hours respectively from the time of dosing in the two study periods.

Meal plan will be identical for both periods of the study.

Activity Level

Subjects should be seated upright for the first 04.00 hours following drug administration. However, in case of an adverse event and at the time of vital signs measurement, subjects will be appropriately positioned. Subjects will be allowed to do normal routine activity avoiding strenuous physical activity during the entire housing period of the study.

Whenever blood sample collection, vital signs examination and meal activities are scheduled at same time, the order of activity followed will be blood sampling, meal and vital signs examination.

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4 SELECTION AND WITHDRAWAL OF SUBJECTS

Subject Screening

Medical history and demographic data including sex, age, body weight (kg), and height (cm), and habits will be recorded during a general screening of volunteers that is organized within 21 days prior to study start. Each subject will undergo a complete general physical examination and laboratory tests for hematology, clinical bio-chemistry, serology, urine analysis, urine pregnancy test and test for alcohol, drugs of abuse as listed in section 5.2. Only medically healthy subjects with clinically acceptable laboratory profiles, chest X-ray (PA-view) and 12-lead ECG will be enrolled into the study.

Clinical/Diagnostic Laboratory Tests

The clinical and laboratory tests performed during screening are tabulated below. All laboratory tests will be performed in Clinical Testing Laboratory, MAKHCRC, Manipal.

For the post-study safety analysis prior to exit from the study, the hematology and clinical bio-chemistry and urine pregnancy test (in case of female subjects only) will be repeated. In case of drop out, the subject will be requested to come for post-study safety analysis.

Laboratory tests performed during the screening are tabulated below.

<table>
<tr><th colspan="3">Clinical / Diagnostic Laboratory Tests</th></tr>
<tr><th>Hematology</th><th>Clinical bio-chemistry</th><th>Urine analysis</th></tr>
<tr><td rowspan="2">▪ Hemoglobin
▪ Hematocrit
▪ Total and differential leukocyte count
▪ Red blood cell count
▪ Platelet count
▪ Erythrocyte Sedimentation Rate (ESR)</td><td>▪ Cholesterol
▪ Random Blood Sugar (RBS)
▪ Creatinine
▪ Sodium
▪ Potassium
▪ Urea</td><td rowspan="2">▪ Color
▪ Transparency
▪ pH
▪ Specific gravity
▪ Protein
▪ Glucose
▪ Ketones
▪ Bilirubin
▪ Blood
▪ Nitrite
▪ Urobilinogen
▪ Microscopic examination</td></tr>
<tr><td>Liver Function Tests (LFTs)
▪ Total Bilirubin
▪ Total proteins
▪ Albumin
▪ Alanine Aminotransferase (ALT)
▪ Aspartate Aminotransferase (AST)
▪ Alkaline Phosphatase (ALP)</td></tr>
<tr><th colspan="2">Serology</th><th>Alcohol, Drugs of Abuse and Urine Pregnancy test</th></tr>
<tr><td colspan="2">▪ Human Immunodeficiency Virus (HIV) 1 and 2
▪ Hepatitis B
▪ Hepatitis C
▪ Rapid Plasma Reagin (RPR)</td><td>▪ Tetrahydrocannabinol (THC)
▪ Opiates
▪ Cocaine
▪ Barbiturates</td></tr>
</table>

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	▪ Amphetamine ▪ Benzodiazepines ▪ Alcohol ▪ Urine pregnancy test (for female volunteers)
Chest X-ray (PA view)	**12-lead Electrocardiogram (ECG)**

Note: Tests for alcohol and drugs of abuse will be performed before check in of each study period. Urine pregnancy test will be performed at time of screening, before check-in of each study period and at the time of period 2 check-out.

Any of the laboratory blood and urine tests as mentioned above may be repeated once with a fresh sample, at the discretion of the Investigator, for confirmation.

Inclusion Criteria

a) Normal healthy human adult female volunteers between 18-45 years (both ages inclusive) of age, who have given written informed consent and are willing to participate in the study

b) Volunteer having Body Mass Index of 18.5 to 29.0 Kg/m^2 (Inclusive).

c) Volunteer with no evidence of underlying disease during the pre-study screening, medical history, physical examination and laboratory investigations performed within 21 days prior to commencement of the study

d) Volunteer whose pre-study screening laboratory tests are either normal or within acceptable limits or are considered by the Investigator to be of no clinical significance with respect to his/her participation in the study.

e) Volunteer with negative test for alcohol, drugs of abuse and urine pregnancy, hepatitis B & C and who is negative or non reactive for antibodies to HIV 1 and 2 and RPR.

f) Volunteer having a 12 lead ECG recording within normal limits.

g) Volunteer with normal chest X-ray taken within 6 months before the day of dosing.

h) Volunteer will be available for the entire study period and is capable of understanding and communicating with the investigators and clinical study facility staff.

Exclusion Criteria

a) Volunteer who is allergic to Levonorgestrel/Ethinylestradiol or any component of the formulation and to any other related drug

b) Volunteers are on any methods of contraception

c) Volunteer with history or having high blood pressure, angina, or heart disease, have had a stroke, have a bleeding or blood-clotting disorder, have breast, uterine, or another hormone-related cancer, have liver disease, have undiagnosed, abnormal vaginal bleeding, have migraines or severe, recurrent headaches, have diabetes, or smoke.

d) Significant cardiovascular, pulmonary, hepatic, renal, hematological, gastrointestinal, endocrine, immunologic, dermatologic, neurological or psychiatric disease.

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- To abstain from consuming alcohol, grapefruit or its juice and cranberry juice for at least 48.00 hours (2 days) prior to administration of drug in period 1 and throughout the study period.
- Not to consume any medication (including over-the-counter products), 14 days preceding administration of drug in period 1 of the study and for the entire duration of study. This restriction includes vitamins taken as nutritional supplements for non-therapeutic indication. If concomitant medication is required in the above mentioned period, the subjects will be treated accordingly, and a decision to continue or discontinue the subjects will be made by the Principal Investigator or Clinical Investigator, based on (a) the time the medication was administered, (b) pharmacology and pharmacokinetic interaction of concomitant medication with the drug.

Discontinuations and Withdrawal

a) Subjects are free to dropout from the study at any time without stating any reason.

b) The investigator can withdraw a subject from the study if:

- The subject suffers from significant inter current illness or undergoes surgery during the course of the study
- The subject requires concomitant medication and the medication has a known and significant drug interaction with the drugs
- The subject experiences adverse event, when withdrawal would be in the best interest of the subject
- The subject fails to comply with the requirements of the protocol
- The subject tests positive for alcohol and/or drugs of abuse and/or urine pregnancy test (for female subjects only).
- It is necessary to further protect the health of the subject or the integrity of the study
- In case of vomiting by a subject after administration of drugs, the subject may be withdrawn based on the investigator opinion.

Termination of the Study

The Principal Investigator reserves the right to discontinue the study for safety reasons at any time.

5 TREATMENT OF SUBJECTS

Dosing

After fasting for at least 10.00 hours, one tablet of Levonorgestrel/Ethinylestradiol will be administered orally to each subject in sitting posture, with 240 ± 2 mL of water at room temperature, in each study period, as per the randomization schedule.

6.4 Concomitant medication

Subjects will not be permitted to take any prescription medicine or Over the Counter (OTC) products (including vitamins and products from natural origin) within 14 days prior to IP administration in period 1 or during the course of the study. If the Principal

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e) Female volunteers who are nursing mothers or are found positive in urine pregnancy test.

f) More specifically, volunteer with history or presence of significant:

- Alcohol dependence, alcohol abuse or drug abuse within the past one year Recent or current alcohol abuse (> 5 units/week, 1 unit= 10 mL or 8 g of pure alcohol) or suspected abuse.
- History of chronic smoking (more than 10 units per day of cigarettes, bidis, or any other form) or chronic consumption of tobacco products.
- Asthma, urticaria or other allergic type reactions after taking any medication.
- Clinically significant illness within 4 weeks before the start of the study.
- Hypersensitivity to heparin.

g) History of clinically relevant allergy (except for untreated, asymptomatic, seasonal allergies at time of dosing) or any allergic reactions to any drugs

h) History of Vascular collapse

i) Volunteer who is scheduled for surgery within 7 days after study completion.

j) Volunteer who, through completion of any other clinical study or otherwise would have donated in excess of 350 mL of blood in the last 90 days.

k) Volunteer who has taken prescription medication or over-the-counter products (including vitamins and products from natural origin) within 14 days prior to administration of drug in period 1, including topical medication meant for systemic absorption

l) Volunteer who was hospitalized within 28 days prior to administration of the study medication.

m) Volunteer with pulse rate less than 60/min or more than 100/min

n) Volunteer with history of difficulty in swallowing

o) Volunteer who has unsuitable veins for repeated venipuncture

p) Any abnormal laboratory value or physical finding which may interfere with the interpretation of test results or cause a health hazard for the volunteer if he participates in the study.

q) Volunteer who has:

- Systolic blood pressure less than 90 mm of Hg or more than 150 mm of Hg
- Diastolic blood pressure less than 60 mm of Hg or more than 94 mm of Hg. Minor deviations (2-4 mm of Hg) at check-in may be acceptable at the discretion of the Investigator.

Restrictions and Prohibitions

Volunteers / Subjects will be instructed/ restricted/prohibited as follows:

- Not to consume tobacco containing products (tobacco chewing, gutkha etc.) and xanthine containing food and beverages, (chocolates, tea, coffee or cola drinks) for at least 48.00 hours (2 days) prior to administration of drug in period 1 and throughout the study period.

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Investigator or Clinical Investigator considers that, some medication is essential for the well-being of the subject, it may be given and if any of the subjects take any medication during the course of the study, they must inform the Principal Investigator or Clinical Investigator. The decision to withdraw a subject from the study will be taken by the Principal Investigator based on the possible interference of the drug with the study or analysis, and on the continuing health of the subject. All occasions of non-study drug intake will be recorded.

6 ASSESSMENT OF EFFICACY/BIOEQUIVALENCE

Analytical Method

Plasma concentrations of Levonorgestrel and Ethinylestradiol will be analyzed using a validated LC-MS/MS method.

Pharmacokinetic parameters & Analysis

Following Pharmacokinetic parameters for Levonorgestrel and Ethinylestradiol will be calculated

C_{max}: Peak concentration, taken as maximum observed concentration in plasma.

t_{max}: Time to peak concentration, taken to be the sampling time at which C_{max} was observed.

AUC_{0-t}: Area under the concentration-time curve from time zero to the last sample with quantifiable concentration calculated using the linear trapezoidal method.

$AUC_{0-\infty}$: Total area under the concentration-time curve from time zero extrapolated to infinity. The area extrapolated to infinity ($AUC_{t-\infty}$) will be obtained by dividing the last quantifiable concentration by the terminal elimination rate constant K_{el}. $AUC_{0-\infty}$ will be determined by adding the extrapolated area $AUC_{t-\infty}$ to AUC_{0-t}. K_{el} will be determined by unweighted linear least-squares regression analysis from the linear segment of the log concentration time data.

$t_{½}$: The terminal elimination half-life calculated by ln2 / K_{el}.

K_{el}: The elimination rate constant

7 ASSESSMENT OF SAFETY

Vital Signs

The supervising physician/clinical investigator or nurse will measure the following vital signs: blood pressure, pulse rate, respiratory rate and oral temperature. Such examination will be done before check in, prior to dosing, on post dose at 02.00, 04.00, 06.00 12.00hrs and during the ambulatory samples in each study period at the scheduled time.

Assessment of the vital signs may also be done, if the attending physician finds it necessary at any time during the conduct of the study and the same will be recorded. In case of any abnormality in vital signs before dosing, medical opinion will be taken as to whether to dose the subject or not.

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Well-being

Subjects will be asked about their well-being before check in, prior to dosing on dosing day and approximately at 02.00, 04.00, 06.00 and 12.00 hours post-dose and during the ambulatory samples in each period of the study. In addition, at all times, subjects may report side effects spontaneously to the investigators. The same will also be recorded in the CRF.

Adverse Event Documentation

All adverse events that are reported will be properly documented. In particular the information required will include: a full description of the event, date and time of onset, date and time of dosing, description of the severity of the event, any treatment or diagnostic steps taken in relation to the event, description of the outcome of the event, judgment by the physician of any relationship of the event to the drug or procedures.

EC Notification by Investigator

Reports of all serious and unexpected (including follow-up information) will be submitted to the EC within 7 working days. Copies of each report and documentation of EC notification and receipt will be kept in the PI's binder.

8 STATISTICS

Statistical analyzes will be performed on plasma concentration values and pharmacokinetic parameters of Levonorgestrel/Ethinylestradiol using appropriate software.

9 ETHICS

Basic Principles

This research will be carried out in accordance with the principles of Declaration of Helsinki (Ethical Principles for Medical Research Involving Human Subjects, revised by the WMA General Assembly, Seoul, October 2008), 'ICH GCP', National Regulations (ICMR Guidelines), 'FDA guidelines & recommendations for BA/BE studies', 'Indian GCP', and 'Schedule Y' of Indian Drugs and Cosmetics Act, and Indian Guidelines for Bioavailability and bioequivalence study (CDSCO).

Institutional Ethics Committee

The Institutional Ethics Committee (University Ethics Committee, Manipal) will review this protocol. The study will not start until the committee has approved the final version of the protocol.

Informed Consent

The written informed consent will be obtained from the volunteers prior to screening and study participation.

During the participation in study , the purpose of the study, a copy of the EC approved informed consent form (which describes the study procedures and the possible potential hazards in non technical terms in conformity with regulatory requirements) will be given and presented to the volunteers in a language they can read, write and understand, prior to check-in.

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The language used in the oral and written information about the trial, including the written informed consent form, will be as non-technical as practical and understandable to the volunteer and the impartial witness (if volunteer is unable to read and write).

Before informed consent is obtained from the volunteer(s), the investigator, or a person designated by the investigator, will provide the volunteer/impartial witness ample time to read the ICF and opportunity to inquire about details of the trial and to decide whether or not to participate in the trial. All questions about the trial will be answered to the satisfaction of the subject.

Prior to a volunteer's participation in the study, the written informed consent form should be signed and personally dated by the volunteer.

If a volunteer is unable to read a witness should be present during the entire informed consent discussion. After the written informed consent form and any other written information to be provided to volunteers, is read and explained to the volunteer, and after the volunteer has orally consented to participate in the study, Volunteer will personally date & sign (if capable of doing so) or will put his/her Left thumb impression and the witness should sign and personally date the consent form. By signing the consent form, the witness attests that the information in the consent form and any other written information was accurately explained to, and apparently understood by, the volunteer, and that informed consent was freely given by the volunteer.

A photocopy of signed ICF will be issued to the subject before check-in.

10 REFERENCES

a) http://www.rxlist.com/alesse-drug.htm#

b) http://www.accessdata.fda.gov

11 APPENDICES

Appendix I a & I b : Informed Consent Form (English III a and Kannada III b)

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APPENDIX I a

INFORMED CONSENT FORM FOR ENROLLMENT

Date of IEC Approval: ___/___/_____ **Volunteer ID: _____**

STUDY TITLE

A OPEN LABEL, TWO PERIOD, SINGLE DOSE, CHRONOPHARMACOKINETIC STUDY OF COMMERCIALLY AVAILABLE ETHINYL ESTRADIOL/ LEVONORGESTREL (0.05MG /0.25MG)TABLETS IN 12 HEALTHY HUMAN ADULT FEMALE SUBJECTS UNDER FASTING CONDITIONS.

- This study involves research
- You are free to participate or reject the participation in this study.
- You will be given an oral presentation. you can clarify your queries/doubts pertaining to the study with the presenter/ investigator.
- Details about this particular study are provided in the following pages. Please read this information carefully and understand.
- If you are agreeing to participate in this study, you have to sign each page of this document and give to study personnel for documentation purpose.
- Photocopy of the same will be given to you for your reference.
- During your participation in the clinical study you act as an independent subject

Signature of the Volunteer: _____

VOLUNTEER INFORMATION SHEET

1. RATIONALE AND OBJECTIVES

The primary objective of this study is to compare the single-dose oral bioavailability of commercially available Ethinylestradiol and Levonorgestrel in healthy human adult female subjects on different time of dosing under fasting conditions.

By conducting this study, on the basis of the data obtained from this study we will know is there any difference in rate and extent of absorption of Ethinylestradiol and Levonorgestrel on different timings of dosing in a day.

2. INVESTIGATIONAL PRODUCT INFORMATION

Ethinyl estradiol and levonorgestrel are forms of estrogen and progesterone, which are both female hormones involved in conception.

Ethinyl estradiol and levonorgestrel are used together in this product as an emergency contraceptive (EC) to prevent pregnancy after contraceptive failure or unprotected intercourse. Ethinyl estradiol and levonorgestrel prevent ovulation (the release of an egg from an ovary), disrupt fertilization (joining of the egg and sperm), and inhibit implantation (attachment of a fertilized egg to the uterus).

3. STUDY PROCEDURE

There will be 12 healthy human adult subjects participating in the study, and the study will be conducted at Manipal AcuNova KH Clinical Research Center, Manipal. If selected, you will be one of the 12 subjects participating in this study.

The study consists of two periods in each of which study drug will be administered.

Each study medicine administration will be separated by a washout period of at least 14 days to ensure that the medicine taken during first period of the study is no longer available in your body in the next period.

During each period of the study you will be housed in the clinical study facility for at least 16hours before administration of the drug and you will continue to stay for at least 24 hours after administration (provided your vital signs examination and enquiry of well-being performed before check-out is satisfactory). Therefore you will be housed for at least 40 hours in each study period at Manipal AcuNova KH Clinical Research Center, Manipal.

You are required to take either one commercially available Ethinylestradiol and Levonorgestrel tablet in each of the two periods. In one period drug will be administered at 8.00AM and in another period drug is dosed at 8.00PM.

You are required to fast at least 10 hours prior to study medicine administration. At the time of study medicine administration, you will receive single dose of test or reference medicine with 240 mL ± 2 mL of water.

You will not be allowed to drink water 1 hour before and 1 hour after study medicine administration. Thereafter, water will be allowed at all times.

You will have to continue fasting for 4 hours following the administration of the study medicine. Thereafter, standardised lunch and snacks will be served at 04.00 and 08.00 hours respectively, after the time of dosing during each study period. Meal plan will be identical in both the study periods.

- **Collection of Blood and Urine Samples**

Signature of the Volunteer: _____

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A total of 19 blood samples will be collected from each subject, in each of the 2 study periods. The 'before dosing' blood sample (1 × 4.0 mL) will be collected at 00.00 hr (within 01.00 hr before administration of study medicine, in the morning of the day of dosing).

The 'after dosing' blood samples (1×4.0 mL) will be collected at 00.25, 00.50, 00.75, 01.00, 01.25, 01.50, 01.75, 02.00, 02.33, 02.67, 03.00, 03.50, 04.00, 08.00, 16.00, 24.00, 48.00 and 72.00hours after dosing in each study period.

All the blood samples up to 16.00 hrs will be collected from an ante-cubital vein and 24.00, 48.00 and 72.00hr samples will be collected by direct vein punctures.

Heparin-lock technique (wherein 0.5 mL of flush solution of Heparin 10 USP units/mL will be injected into the indwelling cannula, after each blood draw) will be used to prevent clotting of blood in the indwelling cannula. Before each blood sample is drawn via indwelling cannula, 0.5 mL of blood will be discarded so as to prevent the saline diluted blood and heparin from interfering with the drug analysis. In case the indwelling cannula is blocked or does not function properly or it must be removed for practical reasons, blood samples will be collected by direct vein puncture.

The total volume of blood drawn including the volume necessary for laboratory tests, the volume of blood discarded before each blood draw and the volume required for post study safety analysis will be 188.00 mL per subject for the entire study as mentioned below:

Volume of each sample: Pre-dose blood sample- 4 mL; Post-dose blood sample(s) 4 mL

Total number of samples: 38 (for both periods of the study)

Volume of discarded blood: 16 mL (0.5 mL discarded when using indwelling cannula, in both periods of the study)

Volume of blood collected during screening: 12 mL

Volume of blood collected for post study safety analysis at the end of the study: 8 mL

The amount of blood collected in the two study periods (exclusive of screening and post study safety analysis) =188 mL

Total amount of blood collected: 188 mL

Note: *You may be required to give extra blood sample for repeat laboratory test(s), if it is beyond acceptable range, at the discretion of the investigator. The volume of blood drawn for this purpose has not been included in the above calculation of total volume of blood drawn.*

As a part of screening procedure, tests for alcohol and drugs of abuse will be performed before check in of each study period. Urine pregnancy test (will be performed at the time of screening and before check in of each period.

Your participation in the study will be completed after collection of 8.00 mL of blood sample and urine sample (for female subjects only) in second period of the study, for post study safety analysis.

During the entire study period you are advised not to donate blood. If in case, you have to, inform investigators before donating.

As a measure of comparison a standard Red Cross blood donation is 450 mL in any 10 week period. You are advised not to give more than 900 mL of blood in next 20 week period.

In order to ensure your safety and well-being during the course of the study, your vital signs (oral temperature, blood pressure, pulse rate and respiratory rate) will be examined and recorded and you will be asked how you feel before check-in, before administration of study medicine and approximately at 02:00, 04:00, 06:00,12:00 and 24:00 hours and during the ambulatory samples after administration of the study medicine.

Signature of the Volunteer: _____

4. RESTRICTIONS

i. You are not supposed to take any medication (prescription or over-the-counter products) during the 14 days preceding the administration of study medicine in period 1 of the study and for the entire duration of the study. This prohibition applies to vitamins taken as nutritional supplements for non-therapeutic indications, as determined by the attending physician. If medicines other than the study medicine specified in the study protocol is required, you will be treated accordingly and the decision to continue or discontinue you in the study will be made by Principal Investigator or Clinical Investigator based on the time of administration of the other medicines and the established scientific and medical data on how the administered medicines interacts with the study medicine and how the body deals with the study medicine.

ii. You are not allowed to consume foods and beverages containing the following substances:

- Xanthine (including chocolates, tea, coffee or cola drinks) for at least 48 hours (2 days) before administration of study medicine in period 1 and throughout the study period.
- Alcohol, grapefruit or its juice and cranberry juice for at least 48 hours (2 days) before administration of study medicine in period 1 and throughout the study period.

iii. You are not allowed to consume tobacco containing products (tobacco chewing, gutkha etc.) for at least 48 hours (2 days) before administration of study medicine in period 1 and throughout the study period.

iv. You will have to maintain the sitting upright posture for first 4 hours after dosing.

V. You should not be on any method of contraception at least one week prior to administration of study medicine in period after administration of the last dose of the study medicine.

5. RISKS, DISCOMFORTS AND PRECAUTIONS ASSOCIATED WITH PARTICIPATION IN THIS STUDY

You may experience some pain and / or swellings in your arm during needle prick and from having blood drawn. The location of vein puncture will be varied from one draw to the next to reduce discomfort. A cannula will also be used to withdraw blood samples. Vein puncture or insertion of a cannula can induce local bruises and phlebitis (inflammation of a vein). These procedures can also occasionally trigger vagal reactions (lightheadedness, fainting). These reactions are usually harmless, or of short duration and limited to a feeling of weakness, accompanied by sweating, slowing of heartbeats, and a decrease in blood pressure.

All medicines may cause side effects, but many people have no, or minor, side effects. Some of these most common side effects persist or become bothersome when using ethinyl estradiol and Levonorgestrel are:

Acne; breast tenderness or enlargement; changes in appetite; changes in sexual interest; changes in weight; dizziness; hair loss; headache; nausea; stomach cramps or bloating; unusual spotting or bleeding; vomiting.

6. ALTERNATIVE PROCEDURES OR TREATMENTS

Since this study is for research purpose and not intended to provide any treatment or other health-related benefit, your alternative is not to participate in this study.

7. BENEFITS

Signature of the Volunteer: _____

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Since you do not require treatment with this drug, you will receive no medical benefit from this study.

8. VOLUNTARY NATURE OF PARTICIPATION

You are entirely free to participate or refuse to participate in this study. Refusal to participate or withdrawal from the study will involve no penalty or loss of medical benefits to which you would otherwise be entitled and your refusal or withdrawal will not affect your selection for future studies.

9. COMPENSATION

A total sum of Rs. 1000/-(Rupees one thousand nine hundred fifty only) will be paid to each subject as compensation for participation after the study.

The Principal Investigator reserves the right to discontinue the entire study for safety reasons, at any time. The Principal Investigator also has the right to withdraw you from the study for medical reasons and for non-compliance/violation of study requirements.

It should be understood that your results cannot be used unless you complete all aspects of the study.

10. CONFIDENTIALITY

The records of your medical history, physical examination, laboratory results and any other information or data generated during this study will be made available to ethics committee.

11. MEDICAL TREATMENT FOR INJURY

If you suffer from any injury related to this study, medical treatment will be provided free of cost by the sub investigator.

12. CONTACTS FOR ADDITIONAL INFORMATION

You can clarify any doubts or questions related to this study by the study staff or physician at the centre. However if you require further information related to this study or your rights as a subject you can contact,

Dr. N. Udupa, Chairman, University Ethics Subcommittee, Manipal (Phone No. +91-820- 2922433)

Dr. S.T Balamurali, Principal Investigator, Manipal AcuNova KH Clinical Research Center, Manipal (Tel: +91-820-2922671, Fax: +91-820-2571999)

Signature of the Volunteer: _____

MANIPAL UNIVERSITY ETHICS COMMITTEE MANIPAL-576 104

VOLUNTEER DECLARATION FORM

Study Title:

A open label, two period, single dose, chronopharmacokinetic study of commercially available Ethinylestradiol/ Levonorgestrel (0.05mg /0.25mg)tablets in 12 healthy human adult female subjects under fasting conditions.

Present Status of the Drug: Ethinylestradiol and Levonorgestrel (0.05mg /0.25mg) tablets are available in the market.

Declaration:

1. I confirm that I have read and understood the information provided in the consent form, which details about the study and have had the opportunity to clarify my doubts.
2. I understand that my participation in the study is voluntary and that I am free to discontinue at any time, without my medical care and legal rights being affected.
3. I understand that my participation may be terminated by the Principal Investigator in the interest and safety of my health.
4. I understand that the sponsor of the study, the others working on the sponsor's behalf, the ethics committee and regulatory authorities will not need my permission to look at my health records both in respect of the current study and any further research that may be conducted in relation to it, even if I discontinue from the study.
5. I understand that my identity will not be revealed or released to third parties or published.
6. I agree to take part in the above study.
7. I agree to abide by the rules and regulations of Manipal AcuNova as long as I stay in the facility.
8. I declare that I had given all the details of the consent to my family members

Signature of the Volunteer: _____

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Informed consent given by:

Name :_____

Sign _____ Date: _____ Time : _____

Address: (Applicable only if there is a change of address)

_____

_____

Or

Left Thumb Impression _____

Impartial Witness: I declare that the information in the consent form and any other written information were accurately explained to and apparently understood by the volunteer and that informed consent was freely given by the volunteer.

Name : _____

Address : _____

_____

Sign _____ Date: _____ Time : _____

Informed consent discussion conducted and queries answered by

Name: _____

Sign & Date: _____

Medical related Queries answered and ICF verified by Principal/Clinical Investigator:

Name: _____

Sign & Date: _____

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MANIPAL-576104

ಅನುಬಂಧ I ಬಿ

<u>ಸೇರ್ಪಡೆಗಾಗಿ ಮಾಹಿತಿಯುತ ಒಪ್ಪಿಗೆ ಪತ್ರ</u>

ಐಇಸಿ ಒಪ್ಪಿಗೆ ದಿನಾಂಕ:____/_____/_____ ಸ್ವಯಂಸೇವಕ ಗುರುತು ಸಂಖ್ಯೆ:_____

ಅಧ್ಯಯನ ಶೀರ್ಷಿಕೆ

12 ಮಂದಿ ಆರೋಗ್ಯವಂತ ವಯಸ್ಕ ಸ್ವಯಂಸೇವಕರಲ್ಲಿ ಆಹಾರ ಸೇವಿಸದ ಸ್ಥಿತಿಯಲ್ಲಿ ತೆರೆದ ಲೇಬಲ್‌ನ, ಎರಡು ಅವಧಿಗಳ, ಏಕ ಡೋಸ್ ನ, ಮಾರುಕಟ್ಟೆಯಲ್ಲಿ ಲಭ್ಯವಿರುವ ಎತಿಂನ್ಯೈಲ್ಸ್ಟ್ರಾದಿಯೋಲ್ ಮತ್ತು ಲೆವೊನೋರ್ಜೆಸ್ಟ್ರೆಲ್ (0.05/0.25 ಎಂ.ಜಿ) ಗುಳಿಗೆಗಳ ಕ್ರೊಸ್ಸೋವರ್ ಫಾರ್ಮಕೋ ಕೈನೆಟಿಕ ಅಧ್ಯಯನ.

- ಇದು ಸಂಶೋಧನೆಯನ್ನೊಳಗೊಂಡ ಅಧ್ಯಯನ
- ನೀವು ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಪಾಲ್ಗೊಳ್ಳಲು ಅಥವಾ ಪಾಲ್ಗೊಳ್ಳುವುದನ್ನು ನಿರಾಕರಿಸಲು ಮುಕ್ತರು.
- ನಿಮಗೆ ಇದನ್ನು ಬಾಯ್ಬರೆ ವಿವರಿಸಲಾಗುವುದು. ನೀವು ನಿಮ್ಮ ಪ್ರಶ್ನೆಗಳು/ಸಂಶಯಗಳನ್ನು ನಿಮಗೆ ವಿವರಿಸಿದವರಲ್ಲಿ / ಸಂಶೋಧಕರಲ್ಲಿ ಪರಿಹರಿಸಿಕೊಳ್ಳಬಹುದು.
- ಈ ಅಧ್ಯಯನಕ್ಕೆ ಸಂಬಂಧಿಸಿದ ವಿವರಗಳನ್ನು ಮುಂದಿನ ಪುಟಗಳಲ್ಲಿ ಕೊಡಲಾಗಿದೆ. ದಯವಿಟ್ಟು ಈ ಮಾಹಿತಿಗಳನ್ನು ಎಚ್ಚರಿಕೆಯಿಂದ ಓದಿ ಮತ್ತು ಅರ್ಥಮಾಡಿಕೊಳ್ಳಿ.
- ಈ ಅಧ್ಯಯನದಲ್ಲಿ ನೀವು ಪಾಲ್ಗೊಳ್ಳಲು ಒಪ್ಪುವುದಾದರೆ, ನೀವು ಈ ದಾಖಲೆಯ ಪ್ರತೀ ಪುಟಕ್ಕೆ ಸಹಿ ಮಾಡಬೇಕು ಮತ್ತು ದಾಖಲಾತಿ ಇರಿಸಿಕೊಳ್ಳುವುದಕ್ಕಾಗಿ ಅದನ್ನು ಅಧ್ಯಯನ ಸಿಬ್ಬಂದಿಗೆ ಕೊಡಬೇಕು.
- ನಿಮ್ಮ ಪರಿಶೀಲನೆಗಾಗಿ ಈ ದಾಖಲೆಯ ಫೋಟೊಕಾಪಿ ಪ್ರತಿಯನ್ನು ನಿಮಗೆ ಒದಗಿಸಲಾಗುತ್ತದೆ.
- ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಪಾಲ್ಗೊಳ್ಳುವ ಅವಧಿಯಲ್ಲಿ ನೀವು ಕೇವಲ ಒರ್ವ ಸ್ವತಂತ್ರ ಪಾಲ್ಗೊಳ್ಳುತ್ತಿರುವ ಸ್ವಯಂಸೇವಕರಾಗಿ ಕಾರ್ಯನಿರ್ವಹಿಸುತ್ತೀರಿ.

ಸಹಿ: _____

ಸ್ವಯಂಸೇವಕ ಮಾಹಿತಿ ಹಾಳೆ

1. ತಾತ್ವಿಕ ಹಿನ್ನೆಲೆ ಮತ್ತು ಉದ್ದೇಶಗಳು

ಮಾರುಕಟ್ಟೆಯಲ್ಲಿ ಲಭ್ಯವಿರುವ ಒಂದು ಎತಿನೈಲ್ಎಸ್ಟ್ರಾಡಿಯೋಲ್ ಮತ್ತು ಲೆವೊನೋರ್ಜೆಸ್ಟ್ರೆಲ್ (0.05/0.25 ಎಂ.ಜಿ) ಗುಳಿಗೆಗಳ ಏಕ ಗುಳಿಗೆಯನ್ನು ಡೋಸನ್ನು ಬಾಯಿಯ ಮೂಲಕ ದಿನದ ಬೇರೆ ಬೇರೆ ಅವಧಿಗಳಲ್ಲಿ ಸೇವಿಸಿದಾಗ ಅದರ ಜೈವಿಕ ಲಭ್ಯತೆ (ಹೀರಿಕೆಯ ದರ ಮತ್ತು ವ್ಯಾಪ್ತಿ) ಯನ್ನು ಆಹಾರ ಸೇವಿಸದ ಸ್ಥಿತಿಯಲ್ಲಿ 12 ಮಂದಿ ಆರೋಗ್ಯವಂತ ವಯಸ್ಕ ಸ್ವಯಂಸೇವಕರಲ್ಲಿ ಹೋಲಿಸಿ ನೋಡುವುದು ಈ ಅಧ್ಯಯನದ ಉದ್ದೇಶ. ಈ ಅಧ್ಯಯನವನ್ನು ಸಂಶೋಧನೆಗಾಗಿ ನಡೆಸಲಾಗುತ್ತಿದೆ.

ಈ ಅಧ್ಯಯನವನ್ನು ನಡೆಸುವುದರಿಂದ, ಅಧ್ಯಯನದಿಂದ ಪಡೆದ ದತ್ತಾಂಶಗಳ ಆಧಾರದ ಮೇಲೆ, ನಮಗೆ ಎತಿನೈಲ್ಎಸ್ಟ್ರಾಡಿಯೋಲ್ ಮತ್ತು ಲೆವೊನೋರ್ಜೆಸ್ಟ್ರೆಲ್ (0.05/0.25 ಎಂ.ಜಿ) ಗುಳಿಗೆ ಯನ್ನು ದಿನದ ಬೇರೆ ಬೇರೆ ಅವದಿಗಳಲ್ಲಿ ಸೆವಿಸಿದಾಗ ಅದರ ಹೀರಿಕೆಯ ದರ ಮತ್ತು ವ್ಯಾಪ್ತಿಯಲ್ಲಿ ಬರುವ ವ್ಯತ್ಯಾಸ ಕಂಡು ಹಿಡಿಯಲು ಸಹಕಾರಿಯಾಗುತ್ತದೆ

2. ಸಂಶೋಧನೆಗೊಳಗಾಗಲಿರುವ ಉತ್ಪನ್ನದ ಮಾಹಿತಿ

ಎತಿನೈಲ್ಎಸ್ಟ್ರಾಡಿಯೋಲ್ ಮತ್ತು ಲೆವೊನೋರ್ಜೆಸ್ಟ್ರೆಲ್ ಇಸ್ಟ್ರೋಜನ್ ಮತ್ತು ಪ್ರೋಜೆಸ್ಟೆರೋನ್ ಗಳ ರೂಪಗಳಾಗಿರುತ್ತವೆ ಮತ್ತು ಇವೆರಡು ಸ್ತ್ರೀ ಹಾರ್ಮೋನ್ ಗಳು ಆಗಿರುತ್ತವೆ.

ಎತಿನೈಲ್ಎಸ್ಟ್ರಾಡಿಯೋಲ್ ಮತ್ತು ಲೆವೊನೋರ್ಜೆಸ್ಟ್ರೆಲ್ ನ್ನು ಗರ್ಬನಿರೋದಕಗಳಾಗಿ ಉಪಯೋಗಿಸಲ್ಪಡುತ್ತದೆ. ಇದನ್ನು ಗರ್ಭ ನಿರೋದಕಗಳ ಅಸಫಲತೆ, ಸುರಕ್ಷತೆ ಇಲ್ಲದ ಸಂಬೋಗ ಸಂದರ್ಬಗಳಲ್ಲಿ ಗರ್ಭ ಧರಿಸುವಿಕೆಯನ್ನು ತಡೆಗಟ್ಟುವುದಕ್ಕೆ ಗರ್ಭ ನಿರೋದಕಗಳಾಗಿ ಉಪಯೋಗಿಸಲ್ಪಡುತ್ತದೆ. ಎತಿನೈಲ್ಎಸ್ಟ್ರಾಡಿಯೋಲ್ ಮತ್ತು ಲೆವೊನೋರ್ಜೆಸ್ಟ್ರೆಲ್ ಅಂಡಾ ಶಯ ದಿಂದ ಅಂಡಾ ಣು ಗಳ ಬಿಡುಗಡೆ ಯನ್ನು ತಡೆ ಗಟ್ಟುತ್ತ ದೆ, ಅಂಡಾಣು ಮತ್ತು ವಿರ್ಯಾಣು ಗಳ ಸೇರುವಿಕೆ ಯನ್ನು ತಡೆ ಗಟ್ಟುತ್ತ ದೆ.

3. ಅಧ್ಯಯನ ಕಾರ್ಯವಿಧಾನ

ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಪ್ರಾಯ ಪ್ರಬುದ್ಧರಾದ 12 ಮಂದಿ ಆರೋಗ್ಯವಂತ ವಯಸ್ಕ ಸ್ವಯಂಸೇವಕರು ಪಾಲ್ಗೊಳ್ಳುತ್ತಿದ್ದಾರೆ ಮತ್ತು ಅಧ್ಯಯನವು ಮಣಿಪಾಲ ಅಕ್ಯುನೋವ ಕೆ ಎಚ್ ಕ್ಲಿನಿಕಲ್ ರಿಸರ್ಚ್ ಸೆಂಟರ್, ಮಣಿಪಾಲದಲ್ಲಿ ನಡೆಯುತ್ತದೆ. ಆಯ್ಕೆಯಾದಲ್ಲಿ, ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಪಾಲ್ಗೊಳ್ಳುವ 28 ಮಂದಿ ಸ್ವಯಂಸೇವಕರಲ್ಲಿ ನೀವೂ ಒಬ್ಬರಾಗಿರುತ್ತೀರಿ.

ಈ ಅಧ್ಯಯನವು ಎರಡು ಅವಧಿಗಳದಾಗಿದೆ ಹಾಗೂ ಪ್ರತಿ ಅವಧಿಯಲ್ಲಿ ಅಧ್ಯಯನ ಔಷಧಿಯನ್ನು ನೀಡಲಾಗುತ್ತದೆ.

ಅಧ್ಯಯನದ ಎರಡು ಅವಧಿಗಳ ನಡುವೆ ಕನಿಷ್ಠ 14 ದಿನಗಳ 'ಔಷಧಿ ನಿಷ್ಕ್ರಿಯ'(wash-out) ಅವಧಿ ಇರುತ್ತದೆ. ಇದರಿಂದಾಗಿ ಮೊದಲ ಹಂತದಲ್ಲಿ ಕೊಡಲಾದ ಔಷಧಿಯ ಅಂಶ ನಿಮ್ಮ ದೇಹದಲ್ಲಿ ಉಳಿದಿಲ್ಲ ಎಂಬುದನ್ನು ದೃಢಪಡಿಸುವುದು ಸಾಧ್ಯವಾಗುತ್ತದೆ.

ಪ್ರತೀ ಅಧ್ಯಯನ ಅವಧಿಯಲ್ಲಿ ಅಧ್ಯಯನ ಔಷಧಿ ಸೇವಿಸುವ ಮೊದಲು ಕನಿಷ್ಠ 16 ಗಂಟೆಗಳ ಕಾಲ ನೀವು ಬಿಎ/ಬಿಇ ಕ್ಲಿನಿಕಲ್ ಅಧ್ಯಯನ ಸೌಕರ್ಯದಲ್ಲಿ ಉಳಿದುಕೊಳ್ಳಬೇಕಾಗುತ್ತದೆ ಮತ್ತು ಔಷಧಿ ಸೇವನೆಯ ಬಳಿಕ ನೀವು ಕನಿಷ್ಠ 24 ಗಂಟೆಗಳ ಕಾಲ (ನಿರ್ಗಮನದ ಮೊದಲು ನಿಮ್ಮ ಜೈವಿಕ ಚಿಹ್ನೆಗಳು ಮತ್ತು ಯೋಗಕ್ಷೇಮದ ಮೇಲ್ವಿಚಾರಣೆ ತೃಪ್ತಿಕರವಾಗಿದ್ದಲ್ಲಿ)ಇಲ್ಲಿ ಉಳಿದುಕೊಳ್ಳುವುದನ್ನು ಮುಂದುವರೆಸಬೇಕಾಗುತ್ತದೆ. ಹಾಗಾಗಿ ನೀವು ಪ್ರತಿ ಅವಧಿಯಲ್ಲಿ ಕನಿಷ್ಠ 40 ಗಂಟೆಗಳ ಕಾಲ ಮಣಿಪಾಲ ಅಕ್ಯುನೋವ ಕೆ ಎಚ್ ಕ್ಲಿನಿಕಲ್ ರಿಸರ್ಚ್ ಸೆಂಟರ್, ಮಣಿಪಾಲದಲ್ಲಿ ಉಳಿದುಕೊಳ್ಳಬೇಕಾಗುತ್ತದೆ.

ಸಹಿ: _____

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ನೀವು ಮಾರುಕಟ್ಟೆಯಲ್ಲಿ ಲಭ್ಯವಿರುವ ಎತಿನ್ಯೊಇಸ್ತ್ರದಿಯೋಲ್ ಮತ್ತು ಲೆವೊನೋರ್ಜೆಸ್ತ್ರೆಲ್ (0.05/0.25 ಎಂ.ಜಿ) ಒಂದು ಗುಳಿಗೆ ಯನ್ನು ಪ್ರತಿ ಅವಧಿಯಲ್ಲಿ ತೆಗೆದುಕೊಳ್ಳಬೇಕಾಗುತ್ತದೆ. ಒಂದು ಅವಧಿಯಲ್ಲಿ ಒಂದು ಗುಳಿಗೆಯನ್ನು ಬೆಳಗ್ಗೆ 8.೦೦ ಗಂಟೆಗೆ ಮತ್ತು ಮತ್ತೊಂದು ರಾತ್ರಿ 8.೦೦ ಗಂಟೆಗೆ ಸೇವಿಸಬೇಗಗುತ್ತದೆ

ಅಧ್ಯಯನ ಔಷಧಿ ಸೇವಿಸುವ ಮುಂಚೆ 10 ಗಂಟೆಗಳ ಕಾಲ ಉಪವಾಸವಿರಬೇಕಾಗುತ್ತದೆ. ಔಷಧಿಯ ಒಂದು ಗುಳಿಗೆ 240 ಎಂಎಲ್ ±2 ಎಂಎಲ್ ನೀರಿನೊಂದಿಗೆ ನೀಡಲಾಗುತ್ತದೆ.

ಅಧ್ಯಯನ ಔಷಧಿ ಸೇವಿಸುವ 1 ಗಂಟೆ ಮೊದಲು ಹಾಗೂ 1 ಗಂಟೆ ಬಳಿಕ ನಿಮಗೆ ನೀರು ಕುಡಿಯಲು ಅನುಮತಿಯಿರುವುದಿಲ್ಲ. ಆ ಬಳಿಕ ಎಲ್ಲಾ ಸಮಯದಲ್ಲಿ ನೀರು ಕುಡಿಯಬಹುದು.

ಅಧ್ಯಯನ ಔಷಧಿ ಸೇವಿಸಿದ ಬಳಿಕ 4 ಗಂಟೆಗಳ ತನಕ ಉಪವಾಸವನ್ನು ಮುಂದುವರೆಸಬೇಕಾಗುತ್ತದೆ. ಆ ಬಳಿಕ ಪ್ರಮಾಣೀಕೃತ ಊಟ/ತಿಂಡಿ/ ಔಷಧಿ ಪಡೆದಲ್ಲಿಂದ ಕ್ರಮವಾಗಿ 4.00, 8.00 ಮತ್ತು 12.00 ಗಂಟೆಗಳಲ್ಲಿ ನೀಡಲಾಗುತ್ತದೆ. ಊಟದ ಯೋಜನೆಯು ಎರಡು ಅವಧಿಗಳಿಗೆ ಒಂದೇ ರೀತಿಯದ್ದಾಗಿರುತ್ತದೆ.

ರಕ್ತ ಹಾಗೂ ಮೂತ್ರ ಮಾದರಿಗಳ ಸಂಗ್ರಹ

ಪ್ರತಿ ಅಧ್ಯಯನ ಅವಧಿಗಳಲ್ಲಿ ಪ್ರತಿ ಸ್ವಯಂಸೇವಕರಿಂದ ಒಟ್ಟು 19 ರಕ್ತಮಾದರಿಗಳನ್ನು ಸಂಗ್ರಹಿಸಲಾಗುತ್ತದೆ. 'ಔಷಧಿ ಸೇವನೆ ಪೂರ್ವ' ರಕ್ತದ ಮಾದರಿಯನ್ನು (1 X 4ಎಮ್ ಎಲ್) 00.00 ಗಂಟೆಗೆ ಸಂಗ್ರಹಿಸಲಾಗುತ್ತದೆ.

'ಔಷಧಿ ಸೇವನೆ ನಂತರ'ದ ರಕ್ತ ಮಾದರಿಯನ್ನು (1x6 ಎಂಎಲ್) ಅಧ್ಯಯನ ಔಷಧಿ ಸೇವನೆಯ ನಂತರ ಮತ್ತು 00.25, 00.50, 00.75, 01.00, 01.25, 01.50, 01.75, 02.00, 02.33, 02.67, 03.00, 03.50, 04.00, 08.00, 16.00, 24.00, 48.00 ಮತ್ತು 72.00 ಗಂಟೆಗಳಿಗೆ ಸಂಗ್ರಹಿಸಲಾಗುತ್ತದೆ.

16.00 ಗಂಟೆ ವರೆಗಿನ ಎಲ್ಲಾ ರಕ್ತ ಮಾದರಿಗಳನ್ನು ನಿಮ್ಮ ಮುಂಗೈಯ ಯಾವುದಾದರೊಂದು ಅಶುದ್ಧ ರಕ್ತನಾಳದಲ್ಲಿರಿಸಲಾಗಿರುವ ಕೆನ್ಯುಲಾದಿಂದ ತೆಗೆಯಲಾಗುವುದು. ನಂತರದ 24.00, 48.00 ಮತ್ತು 72.00 ರಕ್ತದ ಮಾದರಿಯನ್ನು ನೇರವಾಗಿ ಸೂಜಿ ಚುಚ್ಚಿ ತೆಗೆಯಲಾಗುವುದು. ಒಳಗಿರುವ ಕೆನ್ಯುಲೆಯಲ್ಲಿ ರಕ್ತ ಹೆಪ್ಪುಗಟ್ಟುವಿಕೆಯನ್ನು ತಡೆಯಲು ಹಾರ್ಪಿನ್ ಲಾಕ್ ತಂತ್ರವನ್ನು (ಇಲ್ಲಿ 0.5 ಎಂ ಎಲ್ ಹೆಪಾರಿನ್ 10 ಯು ಎಸ್ ಪಿ/ಎಮ್ ಎಲ್ ದ್ರಾವಣವನ್ನು ಪ್ರತೀ ಬಾರಿ ರಕ್ತದ ಮಾದರಿ ತೆಗೆದ ಬಳಿಕ ಕೆನ್ಯುಲಾದ ಒಳಗೆ ಚುಚ್ಚಲಾಗುವುದು) ಬಳಸಲಾಗುತ್ತದೆ. ಪ್ರತೀ ರಕ್ತದ ಮಾದರಿ ತೆಗೆಯುವಾಗ, ರಕ್ತದ ವಿಶ್ಲೇಷಣೆಯಲ್ಲಿ ಕೆನ್ಯುಲೆಯಲ್ಲಿರುವ ಹೆಪಾರಿನ್ ಔಷಧಿ ಅಧ್ಯಯನಕ್ಕೆ ಅಡ್ಡ ಬರದಂತೆ ತಡೆಯಲು 0.5 ಎಮ್ ಎಲ್ ಗಿಂತ ಕಡಿಮೆ ಪ್ರಮಾಣದ ರಕ್ತವನ್ನು ಹೊರಹಾಕಲಾಗುತ್ತದೆ. ಒಂದು ವೇಳೆ ಒಳಗಿರುವ ಕೆನ್ಯುಲೆ ಸಮರ್ಪಕವಾಗಿ ಕಾರ್ಯ ನಿರ್ವಹಿಸದಿದ್ದಲ್ಲಿ ಅಥವಾ ಕಾರ್ಯಾಚರಣೆಯಲ್ಲಿ ಅಡಚಣೆಗಳುಂಟಾಗಿ ಕೆನ್ಯುಲಾವನ್ನು ತೆಗೆಯಲೇ ಬೇಕಾದ ಸಂದರ್ಭಗಳಲ್ಲಿ ರಕ್ತದ ಮಾದರಿಯನ್ನು ನೇರವಾಗಿ ಸೂಜಿ ಚುಚ್ಚಿ ತೆಗೆಯಬೇಕಾಗಬಹುದು.

ಪ್ರಯೋಗಾಲಯ ತಪಾಸಣೆಗಳು, ಪ್ರತೀ ಬಾರಿ ರಕ್ತದ ಮಾದರಿ ತೆಗೆಯುವ ಮುನ್ನ ಹೊರಹಾಕುವ ರಕ್ತದ ಪ್ರಮಾಣ ಮತ್ತು ಅಧ್ಯಯನೋತ್ತರ ವಿಶ್ಲೇಷಣೆಗಳಿಗೆ ಸೇರಿದಂತೆ ತೆಗೆಯಬೇಕಾಗಿರುವ ರಕ್ತದ ಒಟ್ಟು ಪ್ರಮಾಣವು ಇಡೀ ಅಧ್ಯಯನದ ಅವಧಿಯಲ್ಲಿ ಪ್ರತೀ ಸ್ವಯಂಸೇವಕರಿಗೆ ಕೆಳಗೆ ಸೂಚಿಸಲಾಗಿರುವಂತೆ ಸುಮಾರು 188 ಎಮ್ ಎಲ್ ಗಳಷ್ಟಾಗುತ್ತದೆ.

ಪ್ರತಿ ಮಾದರಿಯ ಪ್ರಮಾಣ:	ಔಷಧಿ ಸೇವನೆ ಪೂರ್ವ ರಕ್ತಮಾದರಿ 4 ಎಂ ಎಲ್
	ಔಷಧಿ ಸೇವನೆ ಬಳಿಕದ ರಕ್ತಮಾದರಿ(ಗಳು) 4 ಎಂಎಲ್
ಮಾದರಿಗಳ ಒಟ್ಟು ಸಂಖ್ಯೆ:	38(ಅಧ್ಯಯನದ ಎರಡೂ ಅವಧಿಗಳಿಗೆ)

ಸಹಿ: _____

ಹೊರಹಾಕುವ ರಕ್ತದ ಪ್ರಮಾಣ: 16 ಎಂ ಎಲ್ (0. 5 ಎಂ ಎಲ್, ಒಳಗಿರುವ ಕೆನ್ಯುಲಾ ಬಳಸಿ ಪ್ರತೀ ಮಾದರಿ ತೆಗೆಯುವ ವೇಳೆ, ಅಧ್ಯಯನದ ಎರಡೂ ಅವಧಿಗಳಲ್ಲಿ)

ಸ್ಕ್ರೀನಿಂಗ್ ವೇಳೆ ಸಂಗ್ರಹಿಸಲಾಗುವ ರಕ್ತದ ಮಾದರಿಯ ಪ್ರಮಾಣ: 12 ಎಂ ಎಲ್

ಅಧ್ಯಯನದ ಅಂತ್ಯದಲ್ಲಿ ಸುರಕ್ಷತೆಯ ಮೌಲ್ಯಮಾಪನಕ್ಕಾಗಿ ಸಂಗ್ರಹಿಸಲಾಗುವ ರಕ್ತದ ಪ್ರಮಾಣ : 08 ಎಂ ಎಲ್

ಒಟ್ಟು ಸಂಗ್ರಹಿಸಲಾಗುವ ರಕ್ತದ ಪ್ರಮಾಣ: 188 ಎಮ್ ಎಲ್

ಸೂಚನೆ: ಪುನರ್ ಪ್ರಯೋಗಾಲಯ ತಪಾಸಣೆ(ಗಳಿ)ಗಾಗಿ ಹೆಚ್ಚುವರಿ ರಕ್ತಮಾದರಿಗಳನ್ನು ಕೊಡಬೇಕಾಗಬಹುದು, ಅದು ಸ್ವೀಕಾರ್ಹ ಮಿತಿಗಿಂತ ಹೆಚ್ಚಾಗಿದ್ದಲ್ಲಿ ಸಂಶೋಧಕರ ನಿರ್ಧಾರಕ್ಕೆ ಒಳಪಟ್ಟಿರುತ್ತದೆ. ಈ ಉದ್ದೇಶಕ್ಕಾಗಿ ಸಂಗ್ರಹಿಸಲಾಗುವ ರಕ್ತದ ಪ್ರಮಾಣವು ಈ ಮೇಲಿನ ಒಟ್ಟು ಸಂಗ್ರಹಿಸಲಾದ ರಕ್ತದ ಪ್ರಮಾಣದಲ್ಲಿ ಸೇರಿಕೆಯಾಗಿಲ್ಲ.

ಸ್ಕ್ರೀನಿಂಗ್ ಪ್ರಕ್ರಿಯೆಯ ಅಂಗವಾಗಿ, ಪ್ರತಿ ಅಧ್ಯಯನ ಅವಧಿಯ ಪ್ರವೇಶಾತಿಗೆ ಮೊದಲು ಮದ್ಯಪಾನ ಹಾಗೂ ಮದ್ಯವ್ಯಸನ ಪರೀಕ್ಷೆಗಳನ್ನು ನಡೆಸಲಾಗುತ್ತದೆ. ಸ್ಕ್ರೀನಿಂಗ್ ಸಮಯದಲ್ಲಿ ಹಾಗೂ ಪ್ರತಿ ಅವಧಿಯ ಪ್ರವೇಶಾತಿಗೆ ಮೊದಲು ಮೂತ್ರ ಗರ್ಭಧಾರಣಾ ನಡೆಸಲಾಗುತ್ತದೆ.

ಅಧ್ಯಯನದ ಎರಡನೆಯ ಅವಧಿಯಲ್ಲಿ ಅಧ್ಯಯನೋತ್ತರ ಸುರಕ್ಷಾ ವಿಶ್ಲೇಷಣೆ ಹಾಗೂ ಮೂತ್ರ ಗರ್ಭಧಾರಣಾ ಪರೀಕ್ಷೆ ಗಳಿಗಾಗಿ ಸಂಗ್ರಹಿಸುವ 8.0 ಎಂಎಲ್ ರಕ್ತಮಾದರಿ ಹಾಗೂ ಮೂತ್ರಮಾದರಿ ಸಂಗ್ರಹದೊಂದಿಗೆ ಅಧ್ಯಯನದಲ್ಲಿ ನಿಮ್ಮ ಪಾಲ್ಗೊಳ್ಳುವಿಕೆಯು ಪೂರ್ಣಗೊಳ್ಳುತ್ತದೆ.

ಇಡೀ ಅಧ್ಯಯನದ ಅವಧಿಯಲ್ಲಿ ನೀವು ರಕ್ತದಾನ ಮಾಡಬಾರದೆಂದು ಸಲಹೆ ನೀಡಲಾಗುತ್ತದೆ. ಅಂತಹ್ಪೇನಾದರೂ ಇದ್ದಲ್ಲಿ ನೀವು ರಕ್ತದಾನಕ್ಕೆ ಮುನ್ನ ಮಣಿಪಾಲ ಅಕ್ಯುನೋವ ಕೆ ಏಚ್ ಕ್ಲಿನಿಕಲ್ ರಿಸರ್ಚ್ ಸೆಂಟರ್, ಮಣಿಪಾಲ, ಇವರಿಗೆ ಮಾಹಿತಿ ನೀಡಬೇಕಾಗುತ್ತದೆ.

ನಿಮ್ಮ ಮಾಪನಕ್ಕಾಗಿ ಒಂದು ಹೋಲಿಕೆ ಬೇಕೆಂದಿದ್ದರೆ, ರೆಡ್ ಕ್ರಾಸ್ ನ ರಕ್ತದಾನ ಮಾನದಂಡಗಳನ್ವಯ ಯಾವುದೇ 10 ವಾರಗಳ ಅವಧಿಯಲ್ಲಿ 450 ಎಂ ಎಲ್ ರಕ್ತದಾನ ಮಾಡಬಹುದು. ಹಾಗಾಗಿ ನೀವು ಮುಂದಿನ 20 ವಾರಗಳ ತನಕ 900 ಎಂ ಎಲ್ ಗಿಂತ ಹೆಚ್ಚಿನ ರಕ್ತ ನೀಡುವುದು ಬೇಡವೆಂದು ನಿಮಗೆ ಸೂಚಿಸಲಾಗುತ್ತದೆ.

ಅಧ್ಯಯನ ಅವಧಿಯಲ್ಲಿ ನಿಮ್ಮ ಸುರಕ್ಷೆ ಹಾಗೂ ಯೋಗಕ್ಷೇಮದ ಖಾತರಿಗಾಗಿ ನಿಮ್ಮ ಜೈವಿಕ ಚಿಹ್ನೆಗಳನ್ನು (ಬಾಯಿಯ ಉಷ್ಣತೆ, ರಕ್ತದೊತ್ತಡ, ನಾಡಿ ಬಡಿತ ಮತ್ತು ಉಸಿರಾಟದ ಬಡಿತ) ಪರೀಕ್ಷಿಸಲಾಗುತ್ತದೆ ಹಾಗೂ ದಾಖಲಿಸಲಾಗುತ್ತದೆ ಹಾಗೂ ನಿಮಗೆ ಹೇಗೆ ಅನ್ನಿಸುತ್ತದೆ ಎಂದು ಪ್ರತಿ ಅಧ್ಯಯನ ಅವಧಿಯಲ್ಲಿ ದಾಖಲಾತಿಗೆ ಮೊದಲು, ಅಧ್ಯಯನ ಔಷಧಿ ಸೇವಿಸುವ ಮೊದಲು ಹಾಗೂ ಅಧ್ಯಯನ ಔಷಧಿ ಸೇವಿಸಿದ ಬಳಿಕ ಸುಮಾರು ಅಥವಾ 02.00, 04.00, 06.00, 12.00, 24.00(ಗಂಟೆಗಳು)ಗಂಟೆಗಳಿಗೆ ಹಾಗೂ ನಿರ್ಗಮನ ಸಮಯದಲ್ಲಿ ನಿಮ್ಮನ್ನು ಕೇಳಲಾಗುತ್ತದೆ.

4. ನಿರ್ಬಂಧಗಳು

i. ಅವಧಿ 1ರಲ್ಲಿ ಔಷಧಿ ಸೇವನೆಗೆ 14 ದಿನಗಳ ಮೊದಲು ಮತ್ತು ಔಷಧಿ ನಿಷ್ಕ್ರಿಯ ಅವಧಿ ಸೇರಿದಂತೆ ಇಡಿ ಅಧ್ಯಯನದುದ್ದಕ್ಕೂ ಪಾಲ್ಗೊಳ್ಳುತ್ತಿರುವ ಅಧ್ಯಯನಿತರು ಯಾವುದೇ ಔಷಧಿಗಳನ್ನು ಸೇವಿಸುವಂತಿಲ್ಲ. (ಪ್ರಿಸ್ಕ್ರಿಪ್ಷನ್ ಅಥವಾ ಓವರ್-ದ-ಕೌಂಟರ್ ಉತ್ಪನ್ನಗಳು) ಚಿಕಿತ್ಸೇತರ ಆವಶ್ಯಕತೆಗಳಿಗಾಗಿ ನಿಮ್ಮ ವೈದ್ಯರ ಸಲಹೆಯ ಮೇರೆಗೆ ನೀವು ಸೇವಿಸುತ್ತಿರಬಹುದಾದ ಹೆಚ್ಚುವರಿ ಪೌಷ್ಟಿಕಾಂಶ ರೂಪದ ವಿಟಾಮಿನ್ ಗಳಿಗೆ ಈ ನಿರ್ಬಂಧ ಅನ್ವಯಿಸುತ್ತದೆ. ಅಧ್ಯಯನದ ನಿಯಾಮಾವಳಿಯಲ್ಲಿ ನಿರ್ದಿಷ್ಟಪಡಿಸಿದ ಸಂಶೋಧನಾ ಉತ್ಪನ್ನವನ್ನು ಹೊರತುಪಡಿಸಿ ಇತರ ಔಷಧಿಗಳನ್ನು ತೆಗೆದುಕೊಳ್ಳಬೇಕಾದಲ್ಲಿ, ಅಧ್ಯಯನದಲ್ಲಿ ಪಾಲ್ಗೊಳ್ಳುತ್ತಿರುವ ಅಧ್ಯಯನಿತರು ಮುಂದುವರಿಯುವ ಅಥವಾ ಮುಂದುವರಿಯದೆ ಇರುವ ನಿರ್ಧಾರವನ್ನು ಪ್ರಧಾನ ಸಂ೦ಶೋಧಕರು ಔಷಧಿ ಸೇವಿಸಿದ ಸಮಯ, ಔಷಧಿ ಶಾಸ್ತ್ರ ಹಾಗೂ ದೇಹದಲ್ಲಿ ಔಷಧಿ ಚಲನಾ ಕ್ರಿಯೆಗಳನ್ನು ಆಧರಿಸಿ ನಿರ್ಣಯಿಸಬೇಕಾಗುತ್ತದೆ.

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ii ಈ ಕೆಳಗಿನ ಅಂಶವಿರುವ ಆಹಾರ ಮತ್ತು ಪಾನೀಯ ಸೇವನೆ ನಿಷೇಧಿಸಲಾಗಿದೆ:

- ಕ್ಯಾಂಫೈನ್ಸ್(ಚಾಕಲೇಟ್‌ಗಳು, ಕಾಫಿ,ಟೀ ಮತ್ತು ಕೋಲಾ ಪೇಯಗಳು ಸೇರಿದಂತೆ): ಅವಧಿ 1 ರಲ್ಲಿ ಔಷಧಿ ಸೇವನೆಗೆ ಕನಿಷ್ಠ 48(2 ದಿನಗಳು) ಗಂಟೆಗಳ ಮೊದಲು ಮತ್ತು ಅಧ್ಯಯನ ಅವಧಿಯುದ್ದಕ್ಕೂ.
- ಮದ್ಯಪಾನ, ದ್ರಾಕ್ಷಿಹಣ್ಣು ಅಥವಾ ಅದರ ರಸಗಳು ಹಾಗೂ ಕ್ರ್ಯಾನ್‌ಬೆರಿ ರಸ: ಅವಧಿ 1 ರಲ್ಲಿ ಔಷಧಿ ಸೇವನೆಗೆ ಕನಿಷ್ಠ 48(2 ದಿನಗಳು) ಗಂಟೆಗಳ ಮೊದಲು ಮತ್ತು ಅಧ್ಯಯನ ಅವಧಿಯುದ್ದಕ್ಕೂ.

iii ಅವಧಿ 1 ರಲ್ಲಿ ಔಷಧಿ ಸೇವನೆಗೆ ಕನಿಷ್ಠ 48(2 ದಿನಗಳು) ಗಂಟೆಗಳ ಮೊದಲು ಮತ್ತು ಅಧ್ಯಯನ ಅವಧಿಯುದ್ದಕ್ಕೂ . ತಂಬಾಕು ಉತ್ಪನ್ನಗಳನ್ನು(ತಂಬಾಕು, ಅಗಿಯುವುದು, ಗುಟ್ಕಾ ಇತ್ಯಾದಿ) ಸೇವಿಸುವುದನ್ನು ನಿಷೇಧಿಸಲಾಗಿದೆ.

iv ಔಷಧಿ ಸೇವನೆಯ ನಂತರ ಮೊದಲ 4ಗಂಟೆಗಳ ಕಾಲ ನೇರವಾಗಿ ಕುಳಿತುಕೊಳ್ಳುವ ಭಂಗಿಯನ್ನು ಕಾಯ್ದುಕೊಳ್ಳಬೇಕಾಗುತ್ತದೆ.

v. ಸ್ವಯಂಸೇವಕರು ಗರ್ಭ ದರಿಸಿರಬಾರದು ಮತ್ತು ಅವಧಿ 1 ರಲ್ಲಿ ಔಷಧಿ ಸೇವನೆಗೆ ಕನಿಷ್ಠ ಒಂದು ವಾರ ಮೊದಲಿನಿಂದ ಯಾವುದೇ ಪರ್ಯಾಪ್ತ ಗರ್ಭನಿರೋಧಕ ವಿಧಾನಗಳನ್ನು ಅನುಸರಿಸಿರಬಾರದು ಅಥವಾ ತೆಗೆದುಕೊಂಡಿರಬಾರದು.

5. ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಪಾಲ್ಗೊಳ್ಳುವಲ್ಲಿ ಆಗಬಹುದಾದ ಅಪಾಯಗಳು, ತೊಂದರೆಗಳು ಮತ್ತು ಮುನ್ನೆಚ್ಚರಿಕೆಗಳು

ಸೂಜಿ ಚುಚ್ಚಿ ರಕ್ತ ತೆಗೆಯುವುದರಿಂದ ನಿಮ್ಮ ಕೈಯಲ್ಲಿ ಸ್ವಲ್ಪ ನೋವು ಮತ್ತು / ಅಥವಾ ಊದಿಕೊಳ್ಳುವಂತಹ ಅನುಭವವಾಗಬಹುದು. ಈ ಕ್ಲೇಶಗಳನ್ನು ಕನಿಷ್ಠಗೊಳಿಸಲು ಪ್ರತೀ ಬಾರಿ ರಕ್ತದ ಮಾದರಿ ತೆಗೆಯುವಾಗ ಬೇರೆ ಬೇರೆ ಬಾಗಗಳಲ್ಲಿ ಸೂಜಿ ಚುಚ್ಚಬೇಕಾಗಬಹುದು. ರಕ್ತದ ಮಾದರಿಗಳ ಸಂಗ್ರಹಕ್ಕೆ ಕೆನ್ಯುಲಾವನ್ನು ಸಹ ಬಳಸಲಾಗುತ್ತದೆ. ರಕ್ತನಾಳಗಳಿಗೆ ಕೆನ್ಯುಲಾ ಚುಚ್ಚಿದಾಗ ಆ ಭಾಗದಲ್ಲಿ ಸಣ್ಣ ಗಾಯ, ರಕ್ತನಾಳದ ಉರಿಯೂತದ ಲಕ್ಷಣಗಳು (ಫ್ಲೆಬೈಟಿಸ್) ಕಂಡುಬರಬಹುದು. ಕೆಲವೊಮ್ಮೆ ಈ ಪ್ರಕ್ರಿಯೆಯ ವೇಳೆ ಮಾನಸಿಕ ಪ್ರತಿಕ್ರಿಯೆಯ ಫಲವಾಗಿ ತಲೆ ಧಿಮ್ಮೆನ್ನುವುದು, ತಲೆ ಸುತ್ತುವುದು (ಲೈಟ್ ಹೆಡೆಡ್ ನೆಸ್, ಫೆಯಿಂಟಿಂಗ್) ಕಾಣಿಸಿಕೊಳ್ಳಬಹುದು. ಸಾಮಾನ್ಯವಾಗಿ ಈ ರೀತಿಯ ಪ್ರತಿಕ್ರಿಯೆಗಳು ಅಲ್ಪಕಾಲಿಕ ಮತ್ತು ಹಾನಿ ಇಲ್ಲದವು ಹಾಗೂ ತಾತ್ಕಾಲಿಕವಾಗಿ ದುರ್ಬಲವಾಗಿರುವ ಭಾವನೆಗೆ, ಬೆವರುವಿಕೆ, ಎದೆ ಬಡಿತ ಸ್ವಲ್ಪ ಕಡಿಮೆಯಾಗುವುದಕ್ಕೆ ಮತ್ತು ರಕ್ತದೊತ್ತಡ ಸ್ವಲ್ಪ ಕಡಿಮೆಯಾಗುವುದಕ್ಕೆ ಸೀಮಿತವಾದಂತಹವು.

ಯಾವುದೇ ಔಷಧಿ ಗೆ ಅದರದೇ ಆದ ದುಷ್ಪರಿಣಾಮಗಲಿರುತ್ತವೆ. ಆದರೆ ತುಂಬಾ ಜನ ಏಕ ಡೋಸ್‌ನಲ್ಲಿ ಯಾವುದೇ ದುಷ್ಪರಿಣಾಮಗಳನ್ನು ಅನುಭಸುವಿದಿಲ್ಲ ಅಥವಾ ಸರಳವಾದ ದುಷ್ಪರಿಣಾಮಗಳನ್ನು ಎದುರಿಸುತ್ತಾರೆ. ಎತಿನ್ಯೈಲ್ಎಸ್ಟ್ರಾದಿಯೋಲ್ ಮತ್ತು ಲೆವೊನೋರ್ಜೆಸ್ಟ್ರೆಲ್ ಸೇವಿಸುವುದರಿಂದ ಆಗಾಗೆ, ವರದಿಯಾದ ಹಾನಿಕರ ಘಟನೆಗಳೆಂದರೆ - ವಾಕರಿಕೆ, ಕಿಬ್ಬೊಟ್ಟೆ ನೋವು, ತಲೆನೋವು, ತಲೆಸುತ್ತುವುದು, ಪಸಿವಿನ ಆಸಕ್ತಿಯಲ್ಲಿ ಬದಲಾವಣೆ, ಮಿಲನದ ಆಸಕ್ತಿಯಲ್ಲಿ ಬದಲಾವಣೆ, ತೂಕದಲ್ಲಿ ಬದಲಾವಣ, ತಲೆ ಸುತ್ತುವಿಕೆ, ಕೂದಲು ಕಳೆದುಕೊಳ್ಳುವುದು, ರಕ್ತ ಸ್ರಾವ.

6. ಪರ್ಯಾಯ ಪ್ರಕ್ರಿಯೆಗಳು ಅಥವಾ ಚಿಕಿತ್ಸೆಗಳು

ಈ ಅಧ್ಯಯನವು ಸಂಶೋಧನಾ ಉದ್ದೇಶಗಳಿಗಾಗಿರುವುದರಿಂದ ಹಾಗೂ ಯಾವುದೇ ಚಿಕಿತ್ಸೆ ನೀಡುವುದು ಅಥವಾ ಇತರ ಆರೋಗ್ಯ ಸಂಬಂಧಿ ಲಾಭಗಳನ್ನು ನಿಮಗೆ ಒದಗಿಸಿಕೊಡುವುದು ಈ ಅಧ್ಯಯನದ ಉದ್ದೇಶವಾಗಿಲ್ಲದಿರುವುದರಿಂದ, ನಿಮಗಿರುವ ಪರ್ಯಾಯ ಹಾದಿಯೆಂದರೆ ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಪಾಲ್ಗೊಳ್ಳದಿರುವುದು.

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7. ಲಾಭಗಳು

ನಿಮಗೆ ಈ ಔಷಧಿ ಬಳಸಿ ಚಿಕಿತ್ಸೆ ನೀಡುವುದು ಅಗತ್ಯವಿರುವುದಿಲ್ಲವಾದ್ದರಿಂದ, ನಿಮಗೆ ಈ ಅಧ್ಯಯನದಿಂದ ಯಾವುದೇ ಆರೋಗ್ಯ ಸಂಬಂಧಿ ಲಾಭ ದೊರೆಯುವುದಿಲ್ಲ.

8. ಪಾಲ್ಗೊಳ್ಳುವಿಕೆಯ ಸ್ವಯಂ ಇಚ್ಛೆಯ ಸ್ವರೂಪ

ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಪಾಲ್ಗೊಳ್ಳಲು ಅಥವಾ ಪಾಲ್ಗೊಳ್ಳುವುದನ್ನು ನಿರಾಕರಿಸಲು ತಾವು ಸಂಪೂರ್ಣವಾಗಿ ಸ್ವತಂತ್ರರು. ಪಾಲ್ಗೊಳ್ಳಲು ನಿರಾಕರಿಸಿದರೆ ಅಥವಾ ಪಾಲ್ಗೊಳ್ಳಲು ಹಿಂದೆ ಸರಿದರೆ, ತಮಗೆ ಯಾವುದೇ ದಂಡ ಇರುವುದಿಲ್ಲ ಅಥವಾ ತಮಗೆ ದೊರೆಯಬೇಕಾದ ಯಾವುದೇ ವೈದ್ಯಕೀಯ ಲಾಭಗಳಿಂದ ತಾವು ವಂಚಿತರಾಗುವುದಿಲ್ಲ ಮತ್ತು ನಿಮ್ಮ ನಿರಾಕರಣೆ ಅಥವಾ ಹಿಂದೆ ಸರಿಯುವಿಕೆ ಮುಂದಿನ ಅಧ್ಯಯನದ ವೇಳೆ ತಮ್ಮ ಆಯ್ಕೆಯ ಮೇಲೆ ಪರಿಣಾಮ ಬೀರುವುದಿಲ್ಲ.

9. ಪರಿಹಾರಧನ

ಅಧ್ಯಯನ ಯಶಸ್ವಿಯಾಗಿ ಪೂರ್ಣಗೊಂಡ ಬಳಿಕ ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಸಂಪೂರ್ಣವಾಗಿ ಪಾಲ್ಗೊಳ್ಳುವುದಕ್ಕೆ ಸೂಕ್ತ ಪರಿಹಾರಧನವಾಗಿ ರೂ. 1000/-(ಒಂದು ಸಾವಿರ ರೂಪಾಯಿ ಮಾತ್ರ)ಪ್ರತಿಯೊಬ್ಬ ಸ್ವಯಂಸೇವಕರಿಗೂ ನೀಡಲಾಗುವುದು

ಸುರಕ್ಷಾ ಕಾರಣಗಳಿಗಾಗಿ, ಯಾವುದೇ ಸಮಯದಲ್ಲಿ ಇಡಿ ಅಧ್ಯಯನವನ್ನು ರದ್ದುಗೊಳಿಸುವ ಹಕ್ಕನ್ನು ಪ್ರಧಾನಸಂಶೋಧಕರು ಕಾದಿರಿಸಿಕೊಂಡಿರುತ್ತಾರೆ. ನೀವು ಈ ಅಧ್ಯಯನದ ಎಲ್ಲ ಅಂಶಗಳನ್ನೂ ಕಟ್ಟುನಿಟ್ಟಾಗಿ ಪಾಲಿಸದಿದ್ದಲ್ಲಿ, ನಿಮ್ಮ ಫಲಿತಾಂಶಗಳನ್ನು ಈ ಅಧ್ಯಯನಕ್ಕೆ ಬಳಸಲು ಸಾಧ್ಯವಾಗುವುದಿಲ್ಲ ಎಂಬುದನ್ನು ನೀವು ಅರಿತಿರಬೇಕು.

10. ಗೌಪ್ಯತೆ

ನಿಮ್ಮ ವೈದ್ಯಕೀಯ ವಿವರಗಳು, ದೈಹಿಕ ತಪಾಸಣೆ, ಪ್ರಯೋಗಾಲಯ ಫಲಿತಾಂಶಗಳನ್ನು ಅಥವಾ ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಸಿದ್ಧಗೊಂಡಿರುವ ದತ್ತಾಂಶಗಳನ್ನು ಮತ್ತು ಯಾವುದೇ ವಿವರಗಳನ್ನು ನೈತಿಕ ಅಂಶಗಳ ಸಮಿತಿಗೆ ಒದಗಿಸಬೇಕಾಗಬಹುದು.

11. ಹಾನಿಗಳ ಸಂದರ್ಭದಲ್ಲಿ ಪರಿಹಾರ ಮತ್ತು ವೈದ್ಯಕೀಯ ಚಿಕಿತ್ಸೆ

ಅಧ್ಯಯನದ ಅವಧಿಯಲ್ಲಿ ನಿಮಗೇನಾದರೂ ಹಾನಿಯಾದರೆ, ಅಂತಹ ಸಂದರ್ಭಗಳಲ್ಲಿ ಅಧ್ಯಯನ ನಡೆಸುವವರಿಂದ ಉಚಿತವಾಗಿ ಚಿಕಿತ್ಸೆ ಒದಗಿಸಲಾಗುವುದು.

12. ಹೆಚ್ಚುವರಿ ಮಾಹಿತಿಗಾಗಿ ಸಂಪರ್ಕ

ಈ ಅಧ್ಯಯನದ ಬಗ್ಗೆ ನಿಮಗೇನಾದರೂ ಸಂಶಯಗಳು ಅಥವಾ ಪ್ರಶ್ನೆಗಳು ಇದ್ದಲ್ಲಿ, ಅದನ್ನು ಕೇಂದ್ರದ ಅಧ್ಯಯನ ಸಿಬ್ಬಂದಿ ಅಥವಾ ಫಿಸಿಶಿಯನ್ನರಿಂದ ಪಡೆಯಬಹುದು. ಈ ಅಧ್ಯಯನದ ಬಗ್ಗೆ ಅಥವಾ ಸ್ವಯಂಸೇವಕರಾಗಿ ನಿಮ್ಮ ಹಕ್ಕುಗಳ ಬಗ್ಗೆ ಹೆಚ್ಚುವರಿ ಮಾಹಿತಿಗಳು ಬೇಕಾಗಿದ್ದಲ್ಲಿ ನೀವು ಇವರನ್ನು ಸಂಪರ್ಕಿಸಬಹುದು,

ಡಾ ಎನ್. ಉಡುಪ, ಅಧ್ಯಕ್ಷರು, ಮಾಹೆ ನೈತಿಕ ಅಂಶಗಳ ಉಪಸಮಿತಿ, ಮಣಿಪಾಲ (ದೂರವಾಣಿ. +91-820-2922433)

ಡಾ.ಎಸ್ ಟಿ ಬಾಲಮುರಳಿ, ಪ್ರಧಾನ ಸಂಶೋಧಕರು, ಮಣಿಪಾಲ ಅಕ್ಯುನೋವ ಕೆ ಎಚ್ ಕ್ಲಿನಿಕಲ್ ರಿಸರ್ಚ್ ಸೆಂಟರ್, ಮಣಿಪಾಲ (ದೂರವಾಣಿ +91-820 – 2922671, ಫ್ಯಾಕ್ಸ್: +91-820-2571999)

ಸಹಿ: _____

ಸ್ವಯಂಸೇವಕರ ಘೋಷಣಾ ಪತ್ರ

12 ಮಂದಿ ಆರೋಗ್ಯವಂತ ವಯಸ್ಕ ಸ್ವಯಂಸೇವಕರಲ್ಲಿ ಆಹಾರ ಸೇವಿಸದ ಸ್ಥಿತಿಯಲ್ಲಿ ತೆರೆದ ಲೇಬಲ್‌ನ, ಎರಡು ಅವಧಿಗಳ, ಏಕ ಡೋಸ್ ನ, ಮಾರುಕಟ್ಟೆಯಲ್ಲಿ ಲಭ್ಯವಿರುವ ಎತಿಂಪ್ಯೋಇಸ್ಟ್ರಾದಿಯೋಲ್ ಮತ್ತು ಲೆವೊನೋರ್ಜೆಸ್ಟ್ರೆಲ್ (0.05/0.25 ಎಂ.ಜಿ) ಗುಳಿಗೆಗಳ ಕ್ರೋನೋ ಫಾರ್ಮಕೋ ಕೈನೆಟಿಕ ಅಧ್ಯಯನ.

ಅಧ್ಯಯನ ಔಷಧಿಯ ಹಾಲಿ ಸ್ಥಿತಿ: ಎತಿಂಪ್ಯೋಇಸ್ಟ್ರಾದಿಯೋಲ್ ಮತ್ತು ಲೆವೊನೋರ್ಜೆಸ್ಟ್ರೆಲ್ (0.05/0.25 ಎಂ.ಜಿ) ಗುಳಿಗೆಮಾರುಕಟ್ಟೆಯಲ್ಲಿ ಲಭ್ಯವಿದೆ.

ಘೋಷಣೆ:

1. ನಾನು ಸೇರ್ಪಡೆಗಾಗಿ ಮಾಹಿತಿಯುತ ಒಪ್ಪಿಗೆ ಪತ್ರದಲ್ಲಿರುವ ಅಧ್ಯಯನದ ಬಗೆಗಿನ ಮಾಹಿತಿಗಳನ್ನು ಓದಿ ಅರ್ಥ ಮಾಡಿಕೊಂಡಿದ್ದೇನೆಂದು, ಮತ್ತು ಆ ಬಗೆಗಿನ ಪ್ರಶ್ನೆಗಳನ್ನು ಕೇಳಲು ನನಗೆ ಅವಕಾಶ ದೊರೆತಿದೆಯೆಂದು ಈ ಮೂಲಕ ದೃಢಪಡಿಸುತ್ತೇನೆ.
2. ಈ ಅಧ್ಯಯನದಲ್ಲಿ ನನ್ನ ಭಾಗವಹಿಸುವಿಕೆಯು ಸ್ವಇಚ್ಛೆಯಿಂದ ಆಗಿರುತ್ತದೆ ಮತ್ತು ನನ್ನ ಔಷಧೋಪಚಾರಕ್ಕೆ ಮತ್ತು ನ್ಯಾಯೋಚಿತ ಹಕ್ಕುಗಳಿಗೆ ಯಾವುದೇ ರೀತಿಯ ಧಕ್ಕೆಯಾಗದಂತೆ, ಯಾವುದೇ ಹಂತದಲ್ಲಿ ಹಿಂದೆ ಸರಿಯಲು ನನಗೆ ಮುಕ್ತ ಅವಕಾಶವಿದೆ.
3. ವೈದ್ಯಕೀಯ ಸಂಶೋಧಕರು ನನ್ನ ಆರೋಗ್ಯದ ಸುರಕ್ಷೆಯ ಹಿತಾಸಕ್ತಿಯಿಂದ ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಪಾಲ್ಗೊಳ್ಳುವುದರಿಂದ ನನ್ನನ್ನು ಕೈಬಿಡಬಹುದೆಂದು ನಾನು ಅರಿತಿದ್ದೇನೆ.
4. ಈ ಅಧ್ಯಯನದ ಪ್ರಾಯೋಜಕರು, ಸಂಶೋಧಕರು, ನೈತಿಕ ಅಂಶಗಳ ಸಮಿತಿ ಮತ್ತು ಔಷಧಿ ನಿಯಂತ್ರಣ ಸಂಸ್ಥೆಗಳಿಗೆ; ನಾನು ಪಾಲ್ಗೊಂಡಿರುವ ಅಧ್ಯಯನಕ್ಕೆ ಸಂಬಂಧಿಸಿದ ವೈದ್ಯಕೀಯ ವಿವರಗಳು, ನಾನು ಅಧ್ಯಯನದಿಂದ ಹಿಂದೆ ಸರಿದರೂ ಸಹ ಇವರಿಗೆ ಲಭ್ಯವಾಗಲು ನನ್ನ ಅನುಮತಿ ಬೇಕಾಗಿರುವುದಿಲ್ಲ.
5. ಮೂರನೇ ವ್ಯಕ್ತಿಗೆ ಅಥವಾ ಪ್ರಕಾಶನಕ್ಕೆ ನನ್ನ ಗುರುತನ್ನು ತೋರಿಸಲಾಗುವುದಿಲ್ಲ ಎಂದು ನನಗೆ ತಿಳಿದಿರುತ್ತದೆ.
6. ಮೇಲೆ ಹೇಳಲಾಗಿರುವ ಅಧ್ಯಯನದಲ್ಲಿ ಪಾಲ್ಗೊಳ್ಳಲು ನನಗೆ ಒಪ್ಪಿಗೆ ಇದೆ.
7. ನಾನು ಮಣಿಪಾಲ ಅಕ್ಯುನೋವ ಕೆ ಎಚ್ ಕ್ಲಿನಿಕಲ್ ರಿಸರ್ಚ್ ಸೆಂಟರ್ ಸೌಲಭ್ಯಗಳಲ್ಲಿ ಇರುವಾಗ, ಅವರ ನೀತಿ ನಿಯಮಗಳನ್ನು ಪಾಲಿಸಲು ಒಪ್ಪುತ್ತೇನೆ.
8. ನಾನು ನನ್ನ ಒಪ್ಪಿಗೆಯ ಎಲ್ಲ ವಿವರಗಳನ್ನು ನನ್ನ ಕುಟುಂಬದ ಸದಸ್ಯರಿಗೆ ಕೊಟ್ಟಿದ್ದೇನೆಂದು ಈ ಮೂಲಕ ದೃಢಪಡಿಸುತ್ತೇನೆ

ಸಹಿ: _____

ಪುಟ 7, 8ರಲ್ಲಿ

ಮಾಹಿತಿಯುತ ಒಪ್ಪಿಗೆ ನೀಡಿದವರು:

ಹೆಸರು: _____

ಸಹಿ_____ ದಿನಾಂಕ:_____ ಸಮಯ:_____

ವಿಳಾಸ: (ವಿಳಾಸ ಬದಲಾವಣೆಯಿದ್ದಲ್ಲಿ ಮಾತ್ರ ಅನ್ವಯವಾಗುತ್ತದೆ)

_____

_____

ಅಥವಾ

ಎಡಗೈ ಹೆಬ್ಬೆರಳ ಗುರುತು_____

ನಿಷ್ಪಕ್ಷ ಸಾಕ್ಷಿ: ಒಪ್ಪಿಗೆ ಪತ್ರದಲ್ಲಿನ ಮಾಹಿತಿ ಮತ್ತು ಇತರ ಯಾವುದೇ ಬರವಣಿಗೆ ರೂಪದ ಮಾಹಿತಿಯನ್ನು ಸರಿಯಾಗಿ ವಿವರಿಸಲಾಗಿದೆ ಮತ್ತು ಸ್ವಯಂಸೇವಕರು ಸ್ಪಷ್ಟವಾಗಿ ಅರ್ಥಮಾಡಿಕೊಂಡಿದ್ದಾರೆ ಮತ್ತು ಸ್ವಯಂಸೇವಕರು ಮಾಹಿತಿಯುತ ಒಪ್ಪಿಗೆಯನ್ನು ಸ್ವಪ್ರೇರಿತರಾಗಿ ನೀಡಿರುತ್ತಾರೆ ಎಂದು ನಾನು ಘೋಷಿಸುತ್ತೇನೆ.

ಹೆಸರು: _____

ವಿಳಾಸ:_____

_____

ಸಹಿ_____ ದಿನಾಂಕ:_____ ಸಮಯ:_____

ಮಾಹಿತಿಯುತ ಒಪ್ಪಿಗೆಯ ಚರ್ಚೆಯನ್ನು ನಡೆಸಿದವರು ಮತ್ತು ಪ್ರಶ್ನೆಗಳಿಗೆ ಉತ್ತರ ನೀಡಿದವರು

ಹೆಸರು: _____ ಸಹಿ & ದಿನಾಂಕ:_____

ವೈದ್ಯಕೀಯ ಸಂಬಂಧಿ ಪ್ರಶ್ನೆಗಳನ್ನು ಉತ್ತರಿಸಿದವರು ಮತ್ತು ಐಸಿಎಫ್ ಅನ್ನು ಪರಿಶೀಲಿಸಿದವರು ಪ್ರಧಾನ/ಕ್ಲಿನಿಕಲ್ ಸಂಶೋಧಕರು:

ಹೆಸರು: _____ ಸಹಿ & ದಿನಾಂಕ:_____

ಸಹಿ: _____

APPENDIX-3

FORMULAE USED

Mean: Sum of all values

Number of values

Standard Deviation (S.D (±)):

$$\sqrt{\frac{\sum(x-\bar{x})^2}{(n-1)}}$$

Precision: Coefficient of variation (CV %): Standard deviation / Mean x 100

Accuracy: % Nominal concentration: Concentration found / Nominal concentration x 100

Percent of recovery: Extracted peak area / Unextracted peak area x 100

Mean Percent of Change:

(Mean or corrected mean concentration of stability samples - Mean or corrected mean concentration of comparison samples) / Mean or corrected mean concentration of comparison samples X 100

Percent of change of stock solutions:

(Mean peak response (stability samples) – Mean peak response (comparison sample) / Mean peak response (Comparison samples) X 100

Percentage deviation: Calculated Conc.-Actual Conc. / Actual conc. x100