"ASSAY, IN-VITRO DISSOLUTION STUDY AND HYDROLYTIC STABILITY STUDY OF DILTIAZEM HYDROCHLORIDE FROM MARKETED GEL FORMULATIONS"

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IN

PHARMACEUTICAL ANALYSIS

BY

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CERTIFICATE

This is to certify that the dissertation work entitled "Assay, in-vitro dissolution study and hydrolytic stability study of Diltiazem Hydrochloride from marketed gel formulations" submitted by Upasana R. Gandhi (10MPH310) in partial fulfillment for the award of Master of Pharmacy in "Pharmaceutical Analysis" is a bonafide research work carried out by the candidate at the Department of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University under our guidance. This work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.

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DECLARATION

I hereby declare that the dissertation entitled "Assay, in-vitro dissolution study and hydrolytic stability study of Diltiazem Hydrochloride from marketed gel formulations" is based on the original work carried out by me under the guidance of Dr. Priti J. Mehta, Head of the Department, Department of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University. I also affirm that this work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.

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LIST OF ABBREVIATIONS

Abs.	Absorbance
API	Active Pharmaceutical Ingredient
AR grade	Analytical reagent grade
BP	British pharmacopoeia
Conc.	Concentration
^{0}C	Degree Celsius
D.D.W	Double distilled water
DH	Diltiazem Hydrochloride
EP	European pharmacopoeia
FT- IR Spectra	Fourier transform infrared spectra
g	Gram
GTN	Glyceryl trinitrate
HCl	Hydrochloric acid
HPLC	High Performance Liquid Chromatography
Hr	hour
ICH	International Conference on Harmonisation
LC/MS	Liquid chromatography /mass spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
mg	miligram

Min.	minute
ml	mililitre
NaOH	Sodium Hydroxide
NLT	Not less than
NMT	Not more than
nm	nanometer
PDA	Photo diode array
RBF	Round bottom flask
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
RSD	Relative standard deviation
RT	Retention time
S/N	Signal to noise ratio
SD	Standard deviation
SFC	Supercritical fluid chromatography
TEA	Triethyl amine
TLC	Thin layer chromatography
USP-NF	United state pharmacopoeia-National Formulary
UV-Vis spectra	Ultra-violet - visible spectra
v/v	Volume/ volume
λ_{max}	Maximum wavelength
µ	micro

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ABSTRACT

Diltiazem Hydrochloride is a calcium channel blocker – antihypertensive drug. Its novel dosage form available in the market is Diltiazem Hydrochloride gel used in the treatment of anal fissure. The aim of the present work was to develop and validate an accurate, simple, precise and cost effective UV Visible spectrophotometric method for its estimation in gel formulations. The efficacy of semi-solid dosage form depends on its drug release profile so it is essential to study the in – vitro drug release profile by Franz diffusion cell. In UV-Visible spectrophotometric method, estimation of Diltiazem Hydrochloride was done on its λ_{max} 236nm using double distilled water as a solvent. The calibration curve was found to be linear in the range of 2.5-25µg/ml for Diltiazem Hydrochloride (r^2 =0.998). In-vitro drug release was performed by Franz diffusion cell and maximum % drug release was found to be 82.33% for DILZEM gel and 79.83% for DILTIACT gel at 120min time period. Assay was performed by RP-HPLC method with XTerra C_{18} column (150mm× 4.6mm ,5µm) with mobile phase containing Methanol:Acetonitrile:Ammonium acetate buffer (38:26:36 %v/v/v) at detection wavelength 249nm. The linearity of Diltiazem Hydrochloride was found to be 5-30µg/ml (r^2=0.999). Assay of two marketed formulations was found to be 86.86% for DILZEM gel and 87.43% for DILTIACT gel. Less assay of gel formulations indicates the degradation of the drug so it was necessary to perform degradation study. The hydrolytic degradation study of Diltiazem Hydrochloride API and Gel formulations were carried out at different temperature, time and pH. 1.80% and 6.22% degradation for API was found in acidic and alkaline condition respectively.PDA spectra of both degradation product and DH API were same indicative that this degradation product is des-acetyl diltiazem.

1.1 INTRODUCTION TO DISEASE

Anal fissure is a linear tear in the lining of the distal anal canal below the dentate line. It is a common condition affecting all age groups, but it is particularly seen in young and otherwise healthy adults. The incidence is equal in both sexes.[1]

SYMPTOMS

The classic symptoms are anal pain during or after defecation accompanied by the passage of bright red blood per anus. The pain often is severe and may last for a few minutes during or persist for several hours after defecation. Bleeding from an anal fissure usually is modest and separate from the stool.[2,3]

Symptoms from fissures cause considerable morbidity and reduction in quality of life in otherwise generally young healthy individual.[4]

Fissures may be delineated as acute versus chronic and typical versus atypical. Acute fissures generally resolve within 4 to 6 weeks of appropriate management; therefore, chronic fissures are defined as those producing symptoms beyond 6 to 8 weeks. Chronic fissures have additional physical findings of a sentinel tag at the external apex, exposed internal sphincter muscle, and a hypertrophied anal papilla at the internal apex. Typical fissures are usually in the posterior or anterior midline, and are not associated with other diseases.[5] 10% of women and 1% of men have fissures in the anterior midline.[6] In particular, women who develop symptoms postpartum (accounting for 3% to 11% of all chronic fissures) tend to have anterior fissures.[7,8,9]

In contrast, atypical fissures can occur anywhere in the anal canal, can have a wide variety of findings, and tend to be associated with other diseases, including Crohn's Disease, human immunodeficiency virus (HIV) infection, cancer, syphilis, and tuberculosis.[5]

ETIOLOGY AND PATHOGENESIS

The most widely accepted theory regarding the etiology of anal fissure is that it results from the mechanical forces imposed on the anal canal during the passage of stool. Hard stools are most commonly implicated, but explosive liquid stools can produce the same results. Acute anal fissures that do not heal spontaneously, develop secondary changes to the surrounding tissues that signal the long-standing nature of the condition. Over time, the skin distal to the fissure becomes edematous and enlarged, and may form a fibrous skin tag. In this situation, it is often referred to as a sentinel pile. Similarly, the anal papilla cephalad to the fissure can undergo parallel changes and become enlarged. These changes are attributed to chronic low-grade infection. The edges and the base of the fissure tend to become fibrotic over time.[10]

Alternative theories of pathogenesis leading to the development of chronic fissures have been postulated.

1. Trauma During Pregnancy

Of patients with chronic anal fissures, 11% develop symptoms after childbirth. The risk increases with traumatic deliveries, and the fissures are commonly in the anterior midline.

2. Internal Anal Sphincter Hypertonia

The resting pressure in the anal canal is largely a function of the internal sphincter, which is in a continuous state of partial contraction that is nerve-mediated through a-adrenergic pathways and caused by inherent myogenic tone [11,12]. Relaxation of this smooth muscle occurs automatically in response to rectal distention, the so-called rectoanal inhibitory reflex. Acetylcholine through muscarinic receptors and β adrenergic stimulation mediate relaxation in isolated strips of internal sphincter.[13] The same effect is observed in response to electric field stimulation through a nonadrenergic, noncholinergic neuronal pathway, and nitric oxide has been shown to be the neurotransmitter responsible.[13,14,15,16,17]. The administration of pharmacologic agents that relax the internal anal sphincter, effectively reducing anal canal pressure, can lead to healing in most chronic fissures.[18]

3. Local Ischemia

Chronic anal fissure has been described as an ischemic ulcer. The distal anal canal receives its blood supply from the inferior rectal arteries, branches of the internal pudendal arteries. It has been shown that general anesthesia, sphincterotomy, and the application of topical glyceryl trinitrate (GTN) ointment in patients with anal fissure all lower resting anal pressure, while increasing the local tissue perfusion in the distal anal canal.[19,20,21]

TREATMENT

Treatment for anal fissures is described in Figure 1.1.

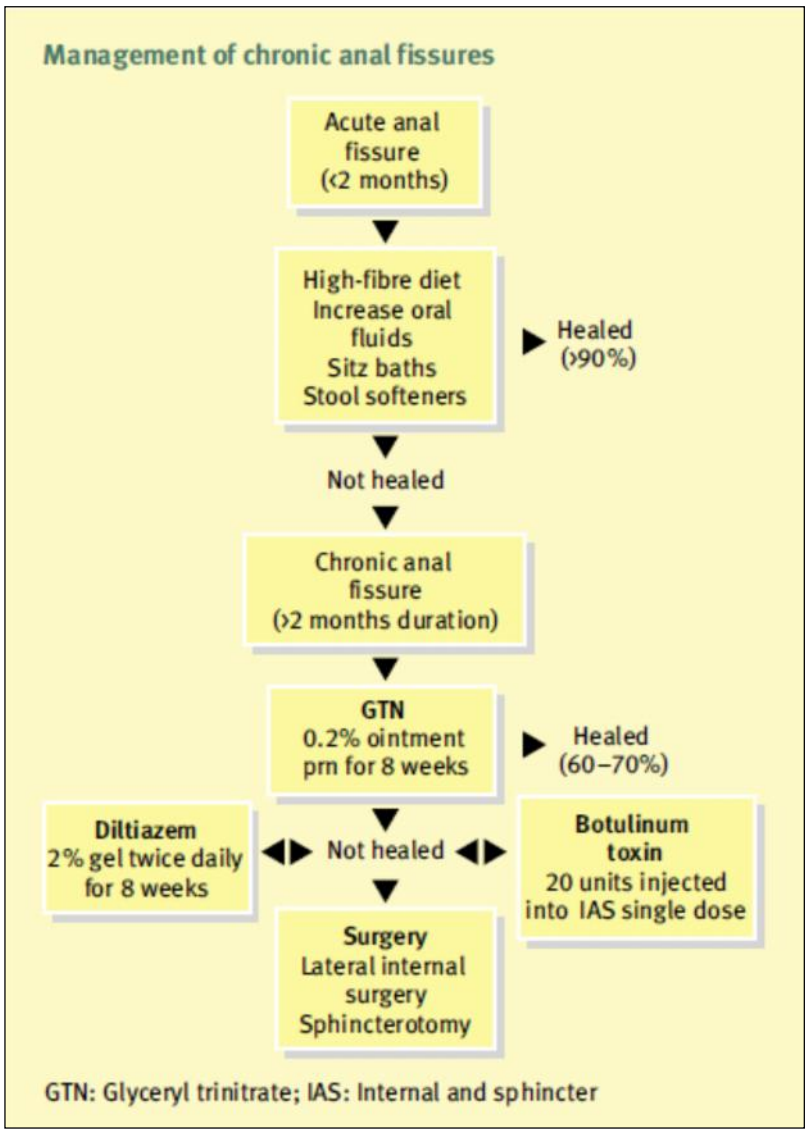

Fig.1.1: Treatment for anal fissure. [22]

(1) ACUTE FISSURE:

A high-fiber diet with an increased intake of water is recommended, laxatives may be used when required to soften constipated stool, and warm sitz baths may offer symptomatic relief.[23]

(2) CHRONIC FISSURE:

The treatment of chronic anal fissure has been directed at reducing the spasm of the internal anal sphincter and anal canal pressure. Traditionally, this reduction has been achieved surgically by manual anal dilation, commonly termed a four-finger anal stretch or internal sphincterotomy, in which the internal anal sphincter is incised. Various pharmacologic agents have been shown to lower resting anal pressure and promote fissure healing. This is so-called chemical sphincterotomy has become accepted as first-line treatment for chronic anal fissure.

- **ORGANIC NITRATES:**

Preparations of isosorbide dinitrate and GTN have been employed successfully, healing a significant proportion of chronic fissures.[24,25,26,27,28,29,30,31,32].These agents act as nitric oxide donors, being metabolized at a cellular level to release this compound. Nitric oxide mediates relaxation of the internal sphincter through the guanylyl cyclase pathway by increasing cyclic guanosine monophosphate levels within the smooth muscle cells.

Topical GTN ointment applied to the anal verge has gained acceptance as an effective treatment for chronic fissures. The dose of GTN delivered depends on the concentration and the volume of ointment applied. A regimen using a pea-sized amount of 0.2% GTN ointment (approximately 0.5 g), applied 2 or 3 times daily to the anal canal for 8 weeks, has been shown to heal two thirds of chronic fissures. [33,34,35,36].The effects of nitrates on the internal anal sphincter are reversible and anal pressures appear to return to pretreatment levels within 3 months of GTN being discontinued after the fissure has healed.[37]

- **CALCIUM CHANNEL BLOCKERS:**

Nifedipine and Diltiazem are prescribed widely in clinical practice as antianginal and antihypertensive agents. They act by blocking slow L-type calcium channels in vascular smooth muscle to cause relaxation and vasodilation. Nifedipine has been shown to reduce lower esophageal sphincter pressure. Io_6 and studies have shown that Nifedipine and Diltiazem decrease resting anal pressure in patients with chronicanal fissure.[38,39,40]

- **α -ADRENOCEPTOR ANTAGONISTS:**

The internal anal sphincter is maintained in a continuous tonic state by the combination of α-adrenergic stimulation and inherent myogenic tone. An oral dose of 20mgof indoramin, an α-adrenoceptor blocker, lowered resting anal pressures.[41]

- **β -ADRENOCEPTOR AGONISTS:**

β -Adrenergic stimulation causes relaxation of internal anal sphincter in vitro and in a preliminary clinical study, Salbutamol- β-adrenoceptor agonist, 4 mg orally, reduced resting anal pressure in volunteers and patients with chronic anal fissures.

- **PARASYMPATHOMIMETICS:**

Bethanechol has been shown to lower resting anal pressures in volunteers and shows promise as an alternative treatment for chronic fissures.[42]

- **BOTULINUM A TOXIN:**

Botulinum toxin A, produced by Clostridium botulinum, is one of the most lethal biologic toxins known to humans but nonetheless has been shown to be valuable in the treatment of various ophthalmologic and neurologic disorders.[43,44,45,46,47] injection of botulinum toxin A into the anal sphincter lowered resting anal pressure and healed 82% of chronic anal fissures, although 6% had recurred by 6 month.[48,49] The treatment itself has a prolonged effect that is nonetheless reversible, avoiding permanent injury to the anal sphincter mechanism. This treatment is invasive and complications such as perianal hematoma, sepsis and pain during injection of the toxin have been reported.

(3) SURGERY:

Although pharmacologic agents may be employed as first-line treatment for chronic fissures, failure of medical therapy in the presence of persistent symptoms warrants surgical intervention. The surgical approaches to chronic fissures have included manual anal dilation or anal stretch, midline sphincterotomy through the base of the fissure later superseded by lateral internal sphincterotomy, and advancement flaps to cover the mucosal defect.[50,51]

Anal stretch is a nonstandardized procedure; the amount of force used as well as degree and duration of stretch varies, with many using four fingers inserted into the anal canal for 4minutes.[52,53,54,55].The resultant disruption to the sphincter mechanism, as shown by endoanal ultrasonography may be significant and lead to permanent damage to the sphincter mechanism.[56,57,58].

Complications after anal dilation include bleeding from the fissure, perianal bruising, perianal infection,Fournier's gangrene,and full thickness rectalprolapse in elderly women.[59,60,61, 62,63,64,65]

Sphincterotomy is superior to anal stretch in terms of healing and, more importantly, poses less of a threat to continence.[66,67]Sphincterotomy initially was performed at the site of the fissure usually in the posterior midline, and the fibers of the internal sphincter were divided in its base.[68,69]Posterior sphincterotomy wounds take noticeably longer to heal than lateral sphincterotomy sites, however and sepsis is more common in the former.[70,71]

Posterior sphincterotomy subsequently was replaced by lateral internal sphincterotomy performed using either a closed technique or under direct vision, under local or general anesthesia.[70, 72,73] Open and closed techniques cause similar reductions in anal pressure and comparable rates of healing; the choice of anesthesia is probably insignificant.[74,75,76]

1.2 DRUG PROFILE:

DILTIAZEM HYDROCHLORIDE [77]

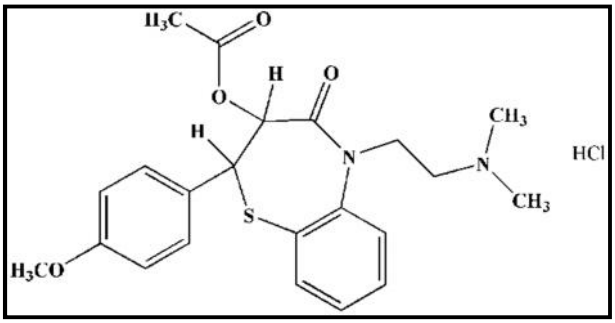

Fig.1.2: Structure of DH.

- IUPAC name : **(**2S-cis)-3-(acetyloxy-5-(2-(dimethylamino)ethyI]-2,3-dihydro-2-(4-methoxy-phenyl)-l,5-benzothia-zepin-4(5H)-one monohydrochloride.
- CAS no : 33286-22-5
- Category : Antihypertensive
- Molecular formula : $C_{22}H_{26}N_20_4S$. HCl
- Molecular mass : 450.98 g/mole
- Appearance : white to off-white crystalline powder, odorless and has a bitter taste.
- LOG (P) : 2.79[78]
- $_P$Ka : 7.7[77]

- Solubility : at 25^0C

Table 1.1: Solubility of DH

SOLVENT	SOLUBILITY
Chloroform	Freely soluble
Formic acid	Freely soluble
Methanol	Freely soluble
Water	Freely soluble
Dehydrated alcohol	Sparingly soluble
Benzene	Practically insoluble
Ether	Insoluble

- Melting point : 207.5^0C to 212^0C

- Official status : IP (2010),USP 31 NF 26, EP 5.0, BP (2010) [79-82]

- Storage : Store protected from light.

- Mechanism of action :

  Diltiazem is a potent vasodilator, increasing blood flow and variably decreasing the heart rate via strong depression of A-V node conduction. t is a potent vasodilator of coronary and peripheral vessels, which reduces peripheral resistance and afterload. Because of its negative inotropic effect, Diltiazem causes a modest decrease in heart muscle contractility and reduces myocardium oxygen consumption. Its negative chronotropic effect results in a modest lowering of heart rate, due to slowing of the sino atrial node. It results in reduced myocardium oxygen consumption.

Because of its negative dromotropic effect, conduction through the AV (atrioventricular) node is slowed, which increases the time needed for each beat. This results in reduced myocardium oxygen consumption.

In anal fissure it acts by blocking slow L-type calcium channels in vascular smooth muscle to cause relaxation and vasodilation.

Table 1.2: Pharmacokinetic parameters of DH [78]

DOSE	(1) Angina pectoris Initial dose of 60mg DTZ three times a day which can be increased to 360mg a day if necessary. (2) Hypertension Initial dose of 60 to 120 mg twice daily which can be increased if necessary,to 360mg/kg daily. (3) Cardiac arrhythmia 0.25mg/kg body weight folled by 0.35mg/kg after 15 min if required. (4) anal fissure 2%gel
DISPOSITION IN THE BODY	Rapidly absorbed (approx,90%) from the GIT and undergoes extensive first-pass hepatic metabolism via desacetylation, n-demethylation, o-demethylation and oxidative deamination.
METABOLITES	A, MA,MB,MX,M1,M2, M4 and M6. 2 to 4% of administered dose appears unchanged in the urine.
BIOAVAILABILITY	About 30 to 40%
HALF LIFE	3 to 7 hr
VOLUME OF DISTRIBUTION	3.1 l/kg for oral administration and 11.1 l/kg intravenously.
DISTRIBUTION IN BLOOD	14 to 23% unbound drug in serum.
PROTEIN BINDING	80 to 85% bound to plasma proteins but only 30 to 40 %bound to albumin.

1.3 INTRODUCTION TO UV-VISIBLE SPECTROMETRY. [83]

The interaction of radiation with matter brings about transitions among some of the quantised energy levels of the matter and study of such an interaction is called spectroscopy.

UV-VIS spectrum results from the interaction of electromagnetic radiation in the UV-VIS region with molecules, ions or complexes. In such determinations the extent to which radiation energy is absorbed by a chemical system as a function of wavelength, as well as, the absorption at a fixed predetermined wavelength of the radiation is measured. Since such measurements need an instrument called spectrometer the technique is known as UV-VIS spectrometry.

The UV-VIS spectrometry is one of the oldest instrumental techniques of analysis and is the basis for a number of ideal methods for the determination of micro and semimicro quantities of analytes in a sample.

ORIGIN OF UV-VIS SPECTRUM:

The absorption of radiation in the UV-VIS region of the spectrum is dependent on the electronic structure of the absorbing species like, atoms, molecules, ions or complexes. The absorption spectrum of atomic species consists of a number of narrow lines that arise as a consequence of the transition amongst the atomic energy levels.

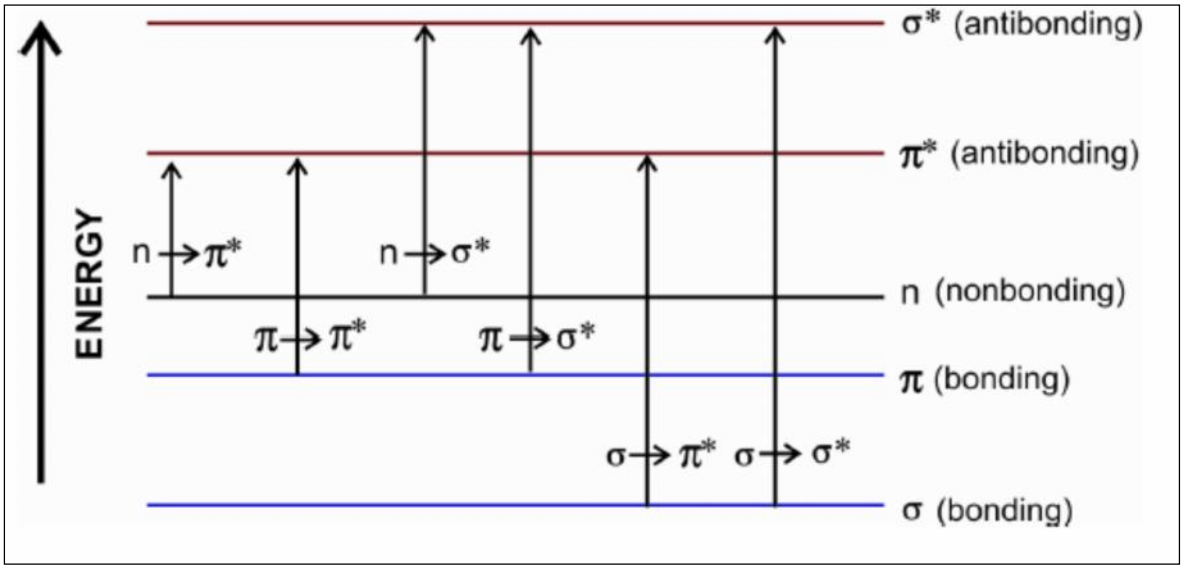

Fig.1.3: Generalized molecular orbital energy level diagram and possible transitions for organic compounds.

In molecules, the electronic, vibrational as well as the rotational energies are quantised. A given electronic energy level has a number of vibrational energy levels in it and each of the vibrational energy level has a number of rotational energy levels in it. When a photon of a given wavelength interacts with the molecule it may cause a transition amongst the electronic energy levels if its energy matches with the difference in the energies of these levels.

CHARACTERISTICS OF UV-VIS SPECTRUM:

In order to obtain a UV-VIS spectrum the sample is ideally irradiated with the electromagnetic radiation varied over a range of wavelength. A monochromatic radiation i.e., a radiation of a single wavelength is employed at a time. This process is called scanning. The amount of the radiation absorbed at each wavelength is measured and plotted against the wavelength to obtain the spectrum. Thus, a typical UV spectrum is a plot of wavelength or frequency versus the intensity of absorption.

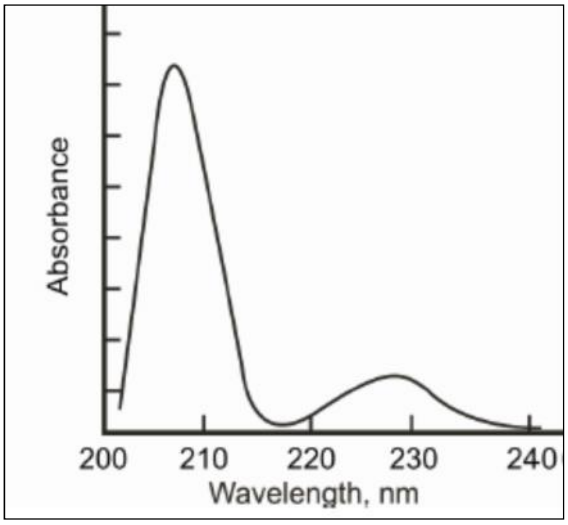

Fig.1.4: Typical UV spectrum.

UV SPECTRUM:

The abscissa (x-axis) indicates the wavelengths absorbed and therefore, is marked in wavelength though sometimes frequency may also be used. The ordinate (y-axis) on the other hand represents the intensity of absorption and is generally represented in terms of absorbance.

The UV spectra of substances are characterized by two major parameters, namely, the position of the maximum of the absorption band called λ_{max}, and the intensity of the bands. The λ_{max} refers to the wavelength of the most absorbed radiation and is a measure of the difference in the electronic energy levels involved in the transition. The intensity on the other hand is indicative of the probability of the transition i.e., whether the transition is allowed or not. It is also is a measure of the concentration of the absorbing species.

The characteristics of UV-VIS spectrum depend on the structure and concentration of the absorbing species in solution. Therefore, these spectra are extensively used in the characterization and in the quantitative estimations of the analyte.

PRINCIPLE OF UV-VIS SPECTROMETRY:

In a typical absorption spectral measurement a monochromatic radiation is made to fall on a sample taken in suitable container called cuvette. In such a situation a part of the radiation is reflected, a part is absorbed, and a part is transmitted. The intensity of original radiation, *Po* is equal to the sum of the intensities of reflected (*P*r), absorbed (*P*a) and transmitted (*P*t) radiation.

Po = Pr + Pa + Pt

The effect of reflection can be compensated by passing equal intensities of beams through the solution and through the solvent contained in the same or similar container and comparing the transmitted radiations. The intensity of the transmitted light is measured and is found to depend on the thickness of the absorbing medium and the concentration, besides the intensity of the incident radiation.

This dependence forms the basis of spectrometric determinations and is given in terms of two fundamental laws. One is Bouguer's law or Lambert's law, which expresses the relationship between the light absorption capacity of the sample and the thickness of the absorbing medium; and the other is Beer's law, which expresses the relationship between the light absorptive capacity of the sample and its concentration. The two laws are combined together to give Beer-Lambert's law.

LAMBERT'S LAW:

Lambert (1760) and Bouguer independently studied the decrease in the intensity of radiation when it passes through a substance and made the following observations:

- The amount of monochromatic light absorbed by a substance is proportional to the intensity of the incident light i.e. the ratio of the intensity of the transmitted and incident light is constant.

- The intensity of the transmitted light decreases exponentially when the thickness of the substance, through which the light is passing, increases linearly. These observations, called Lambert's law can be translated into a mathematical expression as described below.

$$\ln\frac{P}{P_o} = -kb$$ .....eq.(1)

$$\log\frac{P_o}{P} = \frac{k}{2.303}b = k'b$$ .....eq.(2)

P_o represents the radiant power of incident light and P represents the radiant power of transmitted light b represents thickness of the absorbing medium, k is the proportionality constant and the negative sign indicates that radiant power decreases with absorption.

BEER'S LAW:

Beer and Bernard independently studied the dependence of intensity of transmitted light on the concentration of the solution. It was found that the relation between intensity of the transmitted light and concentration was exactly the same as found by Lambert for the intensity of the transmitted light and the thickness of the absorbing medium. Mathematically, the Beer's observations can be expressed as follows:

$$\ln\frac{P}{P_o} = -k^{*}c$$ .....eq.(3)

Or

$$\log_{10}\frac{P_o}{P} = \frac{k^{*}}{2.303}c$$ .....eq.(4)

The Lambert's and Beer's laws are combined and are expressed as

$$\log \frac{P_o}{P} = abc$$ ……,,,,,,,.eq.(5)

In this expression, '*a*' is a constant and is called absorptivity whose value depends on unit of concentration used and is a function of wavelength of the monochromatic light used. The concentration is generally expressed in terms of grams per dm^3 and *b* in cm. Therefore, it has units of $cm^{-1}g\text{-}^{1}dm^3$.

However, if the concentration is expressed as mol $dm^{-3}$and b in cm then it is called molar absorptivity and expressed as ε. Its units are $cm^{-1}mol^{-1}dm^3$. The modified expression for the Beer Lambert's law becomes,

$$\log \frac{P_0}{P} = \varepsilon bc$$ …..……..eq.(6)

The term log *P*o/*P* is called absorbance and is represented as '*A*'.

$$A = \log \frac{P_0}{P}$$ ……..eq.(7)

The expressions for Beer-Lambert's law then becomes

$$\log \frac{P_0}{P} = A = abc \ \ or \ \ \varepsilon bc$$ ……...eq.(8)

The absorbance, *A* is related to another important term called transmittance which is defined as the fraction of the incident radiation transmitted by the absorbing medium.

Mathematically,

$$\text{Transmittance, } T = \frac{P}{P_0}$$ ……eq.(9)

It is generally expressed as a percentage and is expressed as,

$$\text{Percentage transmittance, } \%T = \frac{P}{P_0} \times 100\% \quad \text{.....eq.(10)}$$

It is related to absorbance as,

$$A = -\log T \quad \text{.....eq.(11)}$$

In typical measurements the radiant power transmitted by a solution is measured and compared with that observed with solvent (also called a blank). The ratio of transmitted powers through solution and that through blank or solvent is called as transmittance.

INSTRUMENTATION FOR UV-VIS SPECTROMETRY

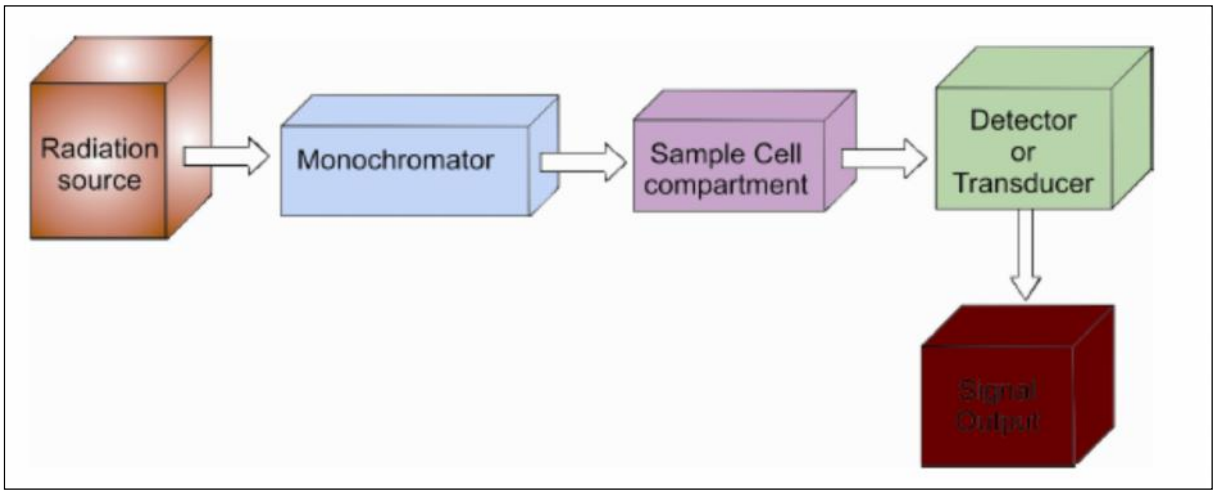

Fig.1.5: Components of UV-Vis instruments.

The five essential components of UV-Vis instruments are as follows

- A stable radiation source
- Wavelength selector
- Sample holder
- Radiation detector or transducer
- Signal processing and output device

ANALYTICAL APPLICATIONS OF UV-VISIBLE SPECTROMETRY:

UV-VIS spectrometry provides a technique that may be used to detect one or more components in a solution and measure the concentration of these species. The primary advantage of this technique is that even traces of substances can be determined in a simple way.

QUALITATIVE APPLICATIONS:

In terms of qualitative analysis of the analyte, the UV-VIS spectrometry is of a secondary importance for the identification and the determination of structural details. The UV spectroscopy has played an important role in the study of a wide array of natural products of plant and animal origin. The changes in spectra due to the changes in the pH of the solution or the solvent can provide useful information about the nature of the analyte.

QUANTITATIVE APPLICATIONS:

It is due its versatility, accuracy and sensitivity. It can be used for direct determination of a large number of organic, inorganic and biochemical species accurately at fairly low concentrations; viz., 10^{-4} to10^{-5} or even lower. In addition to these, the convenience of conducting the determination and its reasonable selectivity make it a method of choice for quantitative determinations.

Another significant feature of this technique is that it may be used for the quantitative determination of analyte which do not absorb in the UV-VIS region. It is achieved by making them react with a reagent that gives a product which absorbs in the region. The same technique can be used to undertake quantitative determination of all such species which have an absorption in the region but are present in system which contain other constituents that also absorb in the same region. Some of the common ones are related to the following quantitative aspects of solution chemistry.

1. Analytical determination of metals and non-metals
2. Analytical determination of organic compounds
3. Determination of dissociation constants of organic acids and dyes
4. Determination of metal-ligand formation constants
5. Determination of kinetic stability of complexes

Quantitative Determination Methodology:

The methodology followed for the quantitative determinations have certain essential steps. These are

- Forming an absorbing species.
- Selection of the measurement wavelength.
- Controlling factors influencing absorbance.
- Validation of Beer's and Lambert's law.

1.4 INTRODUCTION TO HIGH PERFORMANCE LIQUID CHROMATOGRAPHY. [84]

INTRODUCTION

Chromatography is an analytical technique based on the separation of molecules due to differences in their structure and/or composition. In general, chromatography involves moving a sample through the system over a stationary phase. The molecules in the sample will have different affinities and interactions with the stationary support, leading to separation of molecules. Sample components that display stronger interactions with the stationary phase will move more slowly through the column than components with weaker interactions. Different compounds can be separated from each other as they move through the column.

High-performance liquid chromatography (HPLC) is a type of liquid chromatography used to separate and quantify compounds that have been dissolved in solution. HPLC is used to determine the amount of a specific compound in a solution.

CHROMATOGRAPHIC PRINCIPLES

RETENTION:

The retention of a drug with a given packing material and eluent can be expressed as a retention time or retention volume. Retention or elution volume is the quantity of the mobile phase required to pull the sample through the column.

Retention time is defined as how long a component is retained in the column by the stationary phase relative to the time it resides in the mobile phase. The retention is best described as a column capacity ratio (k´), which can be used to evaluate the efficiency of columns. The longer a component is retained by the column, the greater is the capacity factor. The column capacity ratio of a compound (A) is defined by the following equation:

$$k' = \frac{T_A - T_O}{T_O} = \frac{V_A - V_O}{V_O}$$ .....eq.(12)

where V_A is the elution volume of component A and V_0 is the elution volume of a nonretained compound. At constant flow rate, retention times (T_A and T_0) can be used instead of retention or elution volumes.

RESOLUTION:

Resolution is the ability of the column to separate peaks on the chromatograph. Resolution (R) is expressed as the ratio of the distance between two peak maxima to the mean value of the peak width at the base line

$$R = \frac{(T_B - T_A)2}{W_A + W_B} \quad \text{.....eq.(13)}$$

where T_B is the retention time of component B, T_A is the retention time of component A, W_A is the peak width of component A and W_B is the peak width of component B. If R is equal to or more than 1, then components are completely separated, but if R is less than 1, then components overlap.

SENSITIVITY:

Sensitivity is a measure of the smallest detectable level of a component in a chromatographic separation and is dependent on the signal-to-noise ratio in a given detector. Sensitivity can be increased by derivatization of the compound of interest, optimization of chromatographic system or miniaturization of the system.

CHROMATOGRAPHIC MECHANISMS:

Systems used in chromatography are often categorized into one of four types based on the mechanism of action, adsorption, partition, ion-exchange and size exclusion. Adsorption chromatography arises from interactions between solutes and the surface of the solid stationary phase.

Partition chromatography involves a liquid stationary phase that is immiscible with the eluent and is coated on an inert support. Ion exchange chromatography has a stationary phase with an ionically charged surface that is different from the charge of the sample. The technique is based on the ionization of the sample. The stronger the charge of the sample, the stronger the attraction to the stationary phase; therefore, it will take longer to elute off the column.

Size exclusion is as simple as screening samples by molecular size. The stationary phase consists of material with precisely controlled pore size. Smaller particles get caught up in the column material and will elute later than larger particles. Several other types of chromatographic separation have been described, including ion-pair chromatography, which is used as an alternative to ion-exchange chromatography and chiral chromatography (to separate enantiomers).

INSTRUMENTATION:

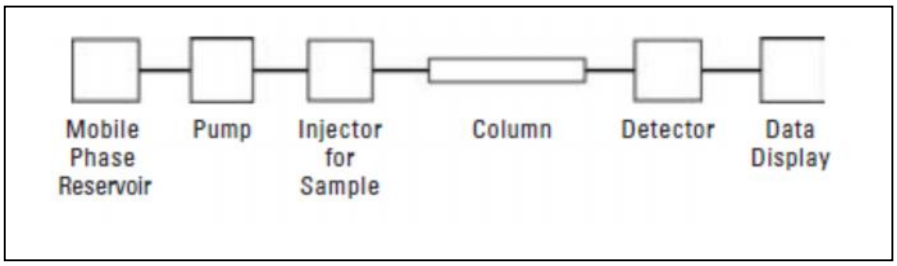

Fig.1.6: Schematic diagram of a High-Performance Liquid Chromatograph.

As shown in the schematic diagram in Figure 1, HPLC instrumentation includes a pump, injector, column, detector and integrator or acquisition and display system. The heart of the system is the column where separation occurs. Since the stationary phase may be composed of micron-sized porous particles, a high-pressure pump is required to move the mobile phase through the column.

The chromatographic process begins by injecting the solute into the injector at the end of the column. Separation of components occurs as the analytes and mobile phase are pumped

through the column. Eventually, each component elutes from the column as a peak on the data display. Detection of the eluting components is important, and the method used for detection is dependent upon the detector used.

The response of the detector to each component is displayed on a chart recorder or computer screen and is known as a chromatogram. To collect, store and analyze the chromatographic data, integrators and other data-processing equipment are frequently used.

MOBILE PHASE AND RESERVOIR:

The type and composition of the mobile phase affects the separation of the components. Different solvents are used for different types of HPLC. For normal-phase HPLC, the solvent is usually nonpolar, and, in reverse-phase HPLC, the solvent is normally a mixture of water and a polar organic solvent. The purity of solvents and inorganic salts used to make the mobile phase is paramount. A general rule of thumb is to use the highest purity of solvent that is available and practical depending on the particular application. The most common solvent reservoirs are as simple as glass bottles with tubing connecting them to the pump inlet.

PUMPS:

High-pressure pumps are needed to push the mobile phase through the packed stationary phase. A steady pump pressure (usually about 1000–2000 psi) is needed to ensure reproducibility and accuracy. Pumps are typically known to be robust, but adequate maintenance must be performed to maintain that characteristic. Inability to build pressure, high pressures or leakage could indicate that the pump is not functioning correctly. Proper maintenance of the pump system will minimize down time.

INJECTORS:

The injector can be a single injection or an automated injection system. An injector for an HPLC system should provide injection of the liquid sample within the range of 0.1-100 ml of volume with high reproducibility and under high pressure (up to 4000 psi). For liquid

chromatography, liquid samples can be directly injected and solid samples need only to be diluted in the appropriate solvent.

DETECTORS:

There are many different types of detectors that can be used for HPLC. The detector is used to sense the presence of a compound passing through and to provide an electronic signal to a data-acquisition device. The main types of detectors used in HPLC are refractive index (RI), ultraviolet (UV-Vis) and fluorescence, but there are also diode array, electrochemical and conductivity detectors.

The recent development of the so-called hyphenated techniques has improved the ability to separate and identify multiple entities within a mixture. These techniques include liquid chromatography-mass spectrometry (LC-MS), liquid chromatography- mass spectrometry-mass spectrometry (LC-MS/MS), liquid chromatography-infrared spectroscopy (LC-IR) and liquid chromatography-nuclear magnetic resonance (LC-NMR). These techniques usually involve chromatographic separation followed by peak identification with a traditional detector such as UV, combined with further identification of the compound with the MS, IR or NMR.

DATA ACQUISITION/DISPLAY SYSTEMS:

Since the detector signal is electronic, use of modern data acquisition techniques can aid in the signal analysis. The data acquisition system of most HPLC systems is a computer. The computer integrates the response of the detector to each component and places it into a chromatograph that is easy to read and interpret. Other more advanced features can also be applied to a chromatographic system. These features include computer-controlled automatic injectors, multi-pump gradient controllers and sample fraction collectors.

COLUMNS:

The column or stationary phase is the core of any chromatographic system. Columns are commercially available in different lengths, bore sizes and packing materials. The use of the correct combination of length and packing material in correlation with the appropriate mobile

phase can assist in the most effective separation of a sample compound. A variety of column dimensions are available including preparative, normal-bore, micro and mini-bore and capillary columns. Different column dimensions can be used for different types of separations and can utilize different packing materials and flow rates.

The most widely used packing materials for HPLC separations are silica-based. The most popular material is octadecyl- silica (ODS-silica), which contains C_{18} coating, but materials with C_1, C_2, C_4, C_6, C_8 and C_{22} coatings are also available. Miscellaneous chemical moieties bound to silica, as well as polymeric packing, are designed for purification of specific compounds.

Other types of column packing materials include zirconia, polymer-based and monolithic columns. Theoretical plates relate chromatographic separation to the theory of distillation and are a measure of column efficiency. The number of theoretical plates (n) can be determined by the following equation

$$n = 16\left(\frac{t_{R_1}}{w}\right)^2 \quad \text{.....eq.(14)}$$

Eluents or with continual injections of “dirty” biological or crude samples column degradation is inevitable, but column life can where tR1 is the total retention time and w is the band width of the peak. In general, LC columns are fairly durable with a long service life unless they are used in some manner that is intrinsically destructive—for example, with highly acidic or basic be prolonged with proper maintenance.

Flushing a column with mobile phase of high elution strength following sample runs is essential. When a column is not in use, it is capped to prevent it from drying out. Particulate samples need to be filtered and when possible a guard column should be utilized. Column regeneration could instill some life into a column, but preventive maintenance is the key to preventing premature degradation.

SEPARATION TECHNIQUES:

ISOCRATIC VERSUS GRADIENT ELUTION:

Elution techniques are methods of pumping mobile phase through a column. In the isocratic method, the composition of the mobile phase remains constant, whereas in the gradient method the composition changes during the separation process. The isocratic method is the simplest technique and should be the first choice when developing a separation. Eluent gradients are usually generated by combining the pressurized flows from two pumps and changing their individual flow rates with an electronic controller or data system while maintaining the overall flow rate constant.

DERIVATIZATION:

Derivatization of samples involves a chemical reaction that alters the molecular structure of the analyte of interest to improve detection. In HPLC, derivatization of a drug is usually unnecessary to achieve satisfactory chromatography. Derivatization is used to enhance the sensitivity and selectivity of detection when available detectors are not satisfactory for the underivatized compounds.

QUANTITATIVE ANALYSIS:

The quantification methods incorporated in HPLC are borrowed mostly from gas chromatography methods. The basic theory for quantitation involves the measurement of peak height or peak area. To determine the concentration (conc.) of a compound, the peak area or height is plotted versus the concentration of the substance. For peaks that are well resolved, both peak height and area are proportional to the concentration. Three different calibration methods, each with its own benefits and limitations, can be utilized in quantitative analysis: external standard (std.), internal standard and the standard addition method.

EXTERNAL STANDARD:

The external standard method is the simplest of the three methods. The accuracy of this method is dependent on the reproducibility of the injection volume. To perform this method, standard solutions of known concentrations of the compound of interest are prepared with one standard that is similar in concentration to the unknown. A fixed amount of sample is injected. Peak height or area is then plotted versus the concentration for each compound. The plot should be linear and go through the origin. The concentration of the unknown is then determined according to the following formula.

$$\text{Conc.}_{\text{unknown}} = \left(\frac{\text{Area}_{\text{unknown}}}{\text{Area}_{\text{known}}}\right)\text{conc.}_{\text{known}} \quad \text{.....eq.(15)}$$

INTERNAL STANDARD:

Although each method is effective, the internal standard method tends to yield the most accurate and precise results. This method, an equal amount of an internal standard, a component that is not present in the sample, is added to both the sample and standard solutions. The internal standard selected should be chemically similar to, have similar retention time and derivatize similarly to the analyte. Additionally, it is important to ensure that the internal standard is stable and does not interfere with any of the sample components. The internal standard should be added before any preparation of the sample so that extraction efficiency can be evaluated. Quantification is achieved by using ratios of peak height or area of the component to the internal standard.

$$\text{Conc.}_{\text{unknown}} = \left(\frac{\text{Area}_{\text{InternalStd.in known}}}{\text{Area}_{\text{InternalStd.in unknown}}}\right) \times \left(\frac{\text{Area}_{\text{unknown}}}{\text{Area}_{\text{known}}}\right) \times (\text{Conc.}_{\text{known}}) \quad \text{.....eq.(16)}$$

VALIDATION:

It is important to utilize a validated LC method when performing analysis. Typical analytical characteristics evaluated in an LC validation include but not are not limited to precision, accuracy, specificity, limit of detection, limit of quantitation, linearity and range.

It is important to consider the US Food and Drug Administration (FDA) and *United States Pharmacopeia-National Formulary* (*USPNF*) guidelines when validating LC methods used for pharmaceutical samples System suitability tests provide an evaluation for the function of the overall LC system. This includes all components that make up a system, such as the instrument, reagents, column packing material, details of the procedure and even the analyst. These tests imply that all of the components of a system constitute a single system in which the overall function can be tested.

System suitability tests are valuable and have been accepted in general application because reliable and reproducible chromatographic results are based on a wide range of specific parameters. In most laboratories there is a standard for the test, at least five replicate injections are made of a single solution that contains 100% of the expected active and excipient ingredients level.

The peak response is measured and the standard deviation of that response should not exceed the limit set by the testing monograph or 2%, whichever of the two is the lowest. Using the USP method, the tailing factors of the analytes should be determined. The values should not exceed 2. Peak-to-peak resolutions are also determined by using the *USP* calculations, and the value should not be lower than 1.5. The system test is used to ensure the quality of the data and of the analysis.

1.5 ANALYTICAL METHOD VALIDATION. [85]

DEFINITION:

Validation is a process of establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics.

Method validation is the process of demonstrating that analytical procedures are suitable for their intended use and that they support the identity, quality, purity, and potency of the drug substances and drug products. Simply, method validation is the process of proving that an analytical method is acceptable for its intended purpose.

A successful Validation guarantees that both the technical and regulatory objectives of the analytical methods have been fulfilled. The transfer of a method is best accomplished by a systematic method validation process. The real goal of validation process is to challenge the method and determine limits of allowed variability for the conditions needed to run the method.

OBJECTIVE OF VALIDATION:

The objective of validation of analytical procedure is to demonstrate that it is suitable for its intended purpose. Validation is documented evidence, which provide a high degree of assurance for specific method. Any developed method may be influenced by variables like different elapsed assay times, different days, reagents lots, instruments, equipments, environmental conditions like temperature, etc so it is expected that after the method has been developed and before it is communicated or transferred from one lab to the other, it is properly validated and the result of validity tests reported.

Two steps are required to evaluate an analytical method.

1) First determine the classification of the method.

2) The second step is to consider the characteristics of the analytical method.

For analytical method validation of pharmaceuticals, guidelines from the International Conference on Harmonization (ICH), United States Food and Drug Administration (US FDA),American Association of Official Analytical Chemists (AOAC)United States Pharmacopoeia (USP), and International Union of Pure and Applied Chemists (IUPAC) provide a framework for performing such validations in a more efficient and productive manner.

The primary objective of validation is to form a basis for written procedure for production and process control which are designed to assure that the drug products have the identity, strength, quality and purity they purport or are represented to possess quality, safety and efficacy must be designed to build into the product. Each step of the manufacturing process must be controlled to maximize the probability that the finished products meet all quality and design specification.

DATA ELEMENTS REQUIRED FOR ASSAY VALIDATION:

Both the USP and ICH recognize that it is not always necessary to evaluate every analytical performance parameter. The type of method and its intended use dictates which parameters needed to be investigated, as illustrated in Table 1.3.

Table 1.3: ICH validation guideline.

TYPE OF ANALYTICAL PROCEDURE	IDENTIFICATION	TESTING FOR IMPURITIES	ASSAY - dissolution (measurement only) - content/potency
characteristics		Quantitat. limit	
Accuracy	-	+ -	+
Precision Repeatability Interm. Precision	 - -	 + - + (1) -	 + + (1)
Specificity (2)	+	+ +	+
Detection Limit	-	- (3) +	-
Quantitation Limit	-	+ -	-
Linearity	-	+ -	+
Range	-	+ -	+

- Signifies that this characteristic is not normally evaluated.

+ Signifies that this characteristic is normally evaluated.

1. Intermediate precision is not needed in some case, when reproducibility is checked.

2. May be needed in some cases.

3. Lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s).

4. May not be needed in some cases.

The different parameters of analytical method development are discussed below as per ICH guideline:

1. SPECIFICITY

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix etc.

2. LINEARITY

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content.

Acceptance criteria:

- The correlation co-efficient (r) value should not be less than 0.995 over the working range.

3. RANGE

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample. The specified range is normally derived from linearity studies and depends on the intended application of the procedure.

4. ACCURACY

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. Several methods of determining accuracy are available:

a) Application of an analytical procedure to an analyte of known purity.

b) Comparison of the results of the proposed analytical procedure with those of a second well characterized procedure, the accuracy of which is stated and/or defined.
c) Accuracy may be inferred once precision, linearity and specificity have been established.

Acceptance criteria:

- Individual and mean % recovery at each level should be 98.0% to 102.0%.

5. PRECISION

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

5.1 Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

Acceptance Criteria:

RSD for assay of six determinations should not be more than 2.0%.

5.2. Intermediate precision

Intermediate precision expresses within-laboratories variations: different days, Different Analysts, different equipment, etc.

Acceptance criteria:

- RSD for assay of six determinations should not be more than 2.0%.
- Difference between the mean assay value obtained in the intermediate precision study and method precision study should not be more than 2.0% absolute.

5.3. Reproducibility

Reproducibility expresses the precision between laboratories. Reproducibility should be considered in case of the standardization of an analytical procedure.

6. DETECTION LIMIT

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. Several approaches for determining the detection limit are possible, depending on whether the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

6.1 Based on Visual Evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods.

6.2 Based on Signal-to-Noise

This approach can only be applied to analytical procedures which exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

6.3 Based on the Standard Deviation of the Response and the Slope

The detection limit (DL) may be expressed as:

$DL = 3.3 \sigma/S$

Where σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimate of σ may be carried out in a variety of ways, for example

6.3.1 Based on the Standard Deviation of the Blank

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

6.3.2 Based on the Calibration Curve

A specific calibration curve should be studied using samples containing an analyte in the range of DL. The residual standard deviation of a regression line or the standard deviation of yintercepts of regression lines may be used as the standard deviation.

Acceptance criteria:

S/N ratio > 3 or 2:1 not specified in other cases.

7. QUANTITATION LIMIT

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. Several approaches for determining the quantitation limit are possible, depending on whether the procedure is a non-instrumental or instrumental.

Approaches other than those listed below may be acceptable.

7.1 Based on Visual Evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods.

7.2 Based on Signal-to-Noise Approach

This approach can only be applied to analytical procedures that exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1.

7.3 Based on the Standard Deviation of the Response and the Slope

The quantitation limit (QL) may be expressed as:

$QL = 10\ \sigma/S$

Where σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimate of σ may be carried out in a variety of ways including:

7.3.1 Based on Standard Deviation of the Blank

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

7.3.2 Based on the Calibration Curve

A specific calibration curve should be studied using samples, containing an analyte in the range of QL. The residual standard deviation of a regression line or the standard deviation of y intercepts of regression lines may be used as the standard deviation.

Acceptance criteria:

- S/N ratio > 10:1; not specified in other cases.

8. ROBUSTNESS

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

Acceptance criteria:

- The difference between assay value of sample analyzed as per test procedure and analysed by applying proposed changes should not be more than 2.0% absolute.

9. SOLUTION STABILITY

Prepare standard and sample as per test procedure and determine initial assay value. Store the standard and sample preparation up to 48 hours at room temperature. Determine the assay of sample preparation after 24 hours and 48 hours storage against freshly prepared standard and determine % response of standard preparation after 24 hours and 48 hours storage against initial standard response. The assay value of sample and % response of standard calculated after 24 hours and 48 hours storage should be compared with the initial value and recorded.

If the stability of solution fails to the acceptance criteria at 24 hour interval at room temperature, repeat the experiment and injecting after standing for 2, 4, 8, 12, and 18 hours at room temperature.

Acceptance criteria:

- The difference in the response of standard preparation should not be more than ± 2.0% from the initial value at any time interval.
- The absolute difference in the assay value of sample should not be more than ± 2.0% from the initial value at each time point.

Table 1.4: Acceptance criteria for validation parameters.

Characteristics	**Acceptance criteria**
Accuracy/trueness	Recovery 98-102%(individual)
Precision	RSD<2%
Repeatability	RSD<2%
Intermediate precision	RSD<2%
Specificity /selectivity	No Interference
Detection limit	S/N>2 or 3
Quantitation limit	S/N >10
Linearity	Correlation coefficient
Range	80-120%
Stability	>24hr or >12hr

SR. NO.	MATRIX	METHOD	DESCRIPTION	REF .NO
1.	Bulk drug, Tablet	LC	**Column:** stainless steel (300mm × 3.9mm,5µm) packed with octadecylsilane bonded to porous silica **Mobile phase:** buffer:Acetonitrile:Methanol (50:25:25) Buffer solution containing 0.116 % w/v of d-10 camphor sulphonic acid in 0.1 M sodium acetate, with the pH adjusted to 6.2 by the addition of 0.1 M sodium hydroxide. **Flow rate:** 1.6ml/min **Detection wavelength**: 240nm **Injection volume**:10µl.	79 &80.
2.	Bulk drug	Potentiometry	Potentiometric titration with 0.1 M perchloric acid.	82.
3.	Tablet, Capsule	Visible spectrophotometry	Includes reaction of the tertiary amino group of the drug with sodium hypochlorite to form the chloro drug derivative. **Detectionwavelength:**540nm **Linearity**-2.5-25.0µg/ml **R^2**=0.9999 **LOD**: 0.12µg/ml **LOQ**:0.37µg/ml	86.
4.	In plasma	HPLC	**Column**: Spherisorb ODS2 reversed-phase column (150mm x 4.6 mm, 5µm) **Mobile phase**: 65% potassium dihydrogen phosphate buffer (0.05 M, pH 3.9) and 35% acetonitrile. **Flow rate**: 1.75 ml/min. **Detection wavelength**:239nm **Linearity range**=10 to 200 ng/ml.	87.

5.	In human serum	HPLC	**Column**: LiChrosorb RP-8 column (300 mm x 4.1 mm I.D., 10 µm) **Mobile phase**: Acetonitrile- 0.01 M dibasic sodium phosphate (40:60v/v) containing 0.1% (v/v) triethanolamine pH was adjusted to 3.0 ± 0.1 with 85% (w/v) orthophosphoric acid. **Detection wavelength**:237nm **Linearity range**:10-500ng/ml **LOD**:2.5ng/ml	88.
6.	Bilk drug, Tablets & Capsule	Spectrophotometric with sodium metavanadate	The drug is boiled with sodium metavanadate in sulfuric acid medium 11.0 M. for 20 min. **Detection wavelength**:750nm $\mathbf{R^2}$=0.999 **Molar absorptivity**: $6.18*10^3$l/mol/cm	89.
7.	Bulk drug	HPLC	**Column**: MicroPak MCH-5 reversed-phase column **Mobile phase**: Mixture of acetonitrile (48%) and water (52%) and contained 0.01 M sodiumn-octanesulfonate. **Detection wavelength**:239nm Exposure of diltiazem aqueous buffered solutions of pH 2 and 7 to UV radiation has a deleterious effect on its stability.	90.

8.	Optical isomers of DH	HPLC	**Column**:Zorbaxsil (150 mm x 4.6 mm I.D.) **Mobile phase A**: dichloromethane-ethyl acetate (100:9) **Mobile phase B**: chloroform-dichloromethane methanoldiethylamine(200:50:30:0.1) **Detection wavelength**:254nm	91.
9.	Enantiomer in dog plasma	Chiral liquid chromatography	**Method A**: **Column**:Nucleosil $5C_{18}$ (150 mm x 4.6 mm I.D.) **Mobile phase**: 0.1% trifluoroacetic acid (TFA)-acetonitrile (7:3, v/v). **Method B**: **Column**:Chiralcel OC (250 mm x 4.6 mm I.D., 5 µm) **Mobilephase**: ethanoldiethylamine (100: 1, v/v). **Detectionwavelength**:240nm **Linearity range**:10 -100ng $\mathbf{R^2}$=0.995 **LOD**: 2ng for l-diltiazem, 3 ng for d-diltiazem.	92.
10.	In human plasma	Automated HPLC	**Column** :Supelcosil LC-18-DB(250mm x 4.6 mm,5µm) **Mobile phase**:Acetonitrile-0.05 M phosphate buffer (pH 2.5) (65:35) containing 0.25% (v/v) triethylamine **Flow rate** : 1 ml/min. **Detection wavelength**:238nm **Linearity** : 10 -1000ng/ml $\mathbf{R^2}$=0.9998 **Intra day precision**:4.3% **Inter day precision**:5.9%	93.

11.	Bulk drug & four metabolites in plasma	HPLC	**Column**:Spherisorb ODS 2 (150 mmx4.6 mm, 3µm) **Mobile phase**:mixture(60 : 40, v/v) of acetonitrile and 0.01 M ammonium phosphate buffer containing 0.06% TEA with pH adjusted to 3.75. **Flow-rate**:1ml/min **Detection wavwlength**:237nm **Linearity** : 10-250ng/ml **Intraday and interday precision**: not more than 12% **LOD**:2ng/ml of plasma **LOQ**:10ng/ml	94.
12.	Optical isomer	HPLC	**Method A**: **Column**: µBondapak C_{18} (300mm x 4 mm I.D.) **Mobile phase**:0.12 M sodium acetate solution containing 1.5 g of d-camphorsulphonic acid (pH-5.5)-acetonitrile-methanol(2:l :l) **Flow rate**:1.6ml/min **Detection wavelength** :240nm **Mthod B:** **Column**: Nucleosil 5C_{18} (200 mm x 4 mm I.D.) **Mobile phase**:0.01M ammonium acetate solution (pH 6.6)-acetonitrile (1:9) **Flow rate** :1.5ml/min **Detection wavelength**:254nm optical purity was more than 99.9 %.	95.

13.	Bulk drug & two metabolites in human plasma	HPLC	**Column** :Spherisorb C8 (100mm X 4.9 mm,5µm) **Mobile phase** :0.005 M phosphate buffer (pH 3.0)-acetonitrile(57:43, v/v) containing 1.25 µM of dibutylamine. **Flow rate** :1.4ml/min **Internal standard**:loxapine (200ng) R^2=0.9978 For D (2.5 to 100ng/ml) R^2=0.9921 for D (100 to 350ng/ml) R^2=0.9921 for MA R^2=0.9983 for DAD **Limit of sensitivity** :2.5ng/ml for D,MA,DAD **LOD**:1ng/ml **Recovery** : over 90% for D and DAD,70 to 75% for MA.	96.
14.	Bulk drug & tablet	Stability indicating HPLC	**Column**:Thermohypersil BDS C_{18},(150mm ×4.6 mm ,5µm) **Mobile phase A:**[0.2%triethylamine (TEA) pH adjustedto4.5with o-phosphoric acid(o-PA)]: **Mobile phase B:** [ACN] in 3:2 (v/v). **Flow rate:**1ml/min **Detection wavelength**:240nm **R^2**: >0.997 for DTZ and six known impurity. **LOD**: below 2% for DTZ and known impurities. Recoveries : (99.8 to 101.2%) for DTZ (97.2 to 101.3%) for six known impurities.	97.
15.	Lipoderm ® transdermal gel	HPLC	**Column** :Atlantis C18 (150mm×4.6mm, 5µm) **Mobile phase**: acetonitrile–sodiumphosphate monobasic monohydrate buffer (pH 2.5, 0.02 M)	98.

			(33:67 v/v) **Flow rate**: 1.0 ml/min **Detector wavelength**:237nm **Linearity range**: 50 to 250 mg/ml $\mathbf{R^2}$=0.996±0.03(n=4) **Inter-day recoveries** ranging from 84.00 to 96.52% **RSD**= 12.01 to 15.94%. **Intra-day recoveries** ranged from 67.95 to 106.1% RSD=<5%	
16.	DH & desacetyl-diltiazem in plasma	Capillary zone electro-phoresis	Sample preparation was performed by liquid –liquid extraction. **Linearity range**: 5-250 ng/ml with veramil as internal standard. $\mathbf{R^2}$=0.999 Precision and accuracy are better than 13%at 5ng/ml, and better than 10%between 10 and 250 ng/ml.	99.
17.	Bulk drug & tablet	HPLC	**Column** :(1) Chiralcel OC, OG, OD and OF columns (250 x 4.6 mm , 10 µm particle size). (2) Ultron ES-OVM (150 x 4.6 mm, 5 µm particle size). **Mobile phase**:hexane and 2-propanol and diethylamine was added (<1%). **Flow rate**:1ml/min **Detection wavelength**:254nm $\mathbf{R^2}$=1.0 for three optical isomer. **LOD**:0.01% **LOQ**:0.05%	100.

18.	In rat plasma	HPLC	**Column** :two 5-µm BDS silica gel column(150mm x 4.6 mm) **Mobile phase**: ammonia solution: methanol: dichloromethane: hexane(0.3:35:30:370) **Flow rate:**2ml/min **Detectionwavelength**:240nm **Linearity range**: 0-800ng/ml of rat plasma **R ^{2}**=0.999 **Intra/interdayprecision**:wthin 5% **Recoveris** in plasma varied from 101.1% at 20ng/ml to 93.7% at 400ng/ml.	101.
19.	Bulk drug	HPLC	Photostability & phototoxocity study was performed. **Column** :RP-18 BondclonePhenomenex (300×3.9 mm) **MS** **Ionizing voltage**:70eV **Mobile phase**: TEA acetate buffer (pH 4.0 0.01M) acetonitrile 72:28(v/v) **Flow rate**:1.4ml/min **Detection wavelength:**220,240,275nm	102.
20.	Tablet	Near – infrared FT-raman spectroscopy,HPLC	**Laser wavelength:**1.064-µm **Laser power:300Mw** **HPLC METHOD** **Column** :LiChrospher 100 RP C_{18}(250mm×4mm, **Flow rate:**1.5ml/min (1)For commercial tablet **HPLC**: 63.57±0.13mg	103.

			RAMAN:63.28±0.26mg (2) Experimental tablet **HPLC**:181.20±0.25mg **RAMAN**:181.22±0.35mg	
21.	Racemic DH,8-chloro derivative of diltiazem, desacetyl form	HPLC	**Column :**Ultron ES-OVM ovomucoidchiral column (150mm×4.6mm,5µm) **Mobile phase:Acetonitrile**-0.02M phosphate buffer pH 6.0 **Flow rate**:1ml/min **Injection volume**:5µl **Column temperature:**40 ^{0}C **Detection wavwlength:**240nm Separations of enantiomers were within 20mins.	104.
22.	Optical isomers of DH	Packed column super-critical fluid chromatography	**Column** : (1) Chiralcel OC, OG, OD and OF columns (250 x 4.6 mm , 10 µm). (2) Ultron ES-OVM (150 x 4.6 mm, 5 µm particle size). **Mobile phase**: Hexane and 2-propanol and diethylamine was added (<1%). **Flow rate**:1ml/min **Detection wavelength**:254nm In p-SFC, four optical isomers achieved the baseline resolution within 8 rain under the higher column efficiency on the Chiralcel OD column, but, in HPLC, *d-trans* and *l-cis* isomers did not resolve on the column.	105.
23.	Bulk drug	Kinetic spectrophotometric	**Reagents :** Ammonium ferric sulphate, hydroxylamine hydrochloride.	106.

24.	Bulk drug	HPLC, HPLC/MS	**HPLC** **Column** : (1) RP-18 Lichrospher(125×4.6mm,5µm) (2) RP-18 Lichrosphere (3) RP-8 Lichrospher(250×4.6mm,5µm) (4) RP-18 Chromolith(100×4.6mm,5µm) Na2HPO4 pH 8.5 + 0.2% DA/ACN (60/40) for (1) ACN/EtOH/KH2PO4 pH 4.5 +DO 25/5/70 for (1) Ammonium acetate pH 6.58 + 0.2% DA/ACN (60/40) for (2) Ammonium acetate pH 6.58 + 0.2% DA/ACN (60/40) for (3) Ammonium acetate + 0.2% DA pH 6.58/ACN (60/40) for (4) **Detection wavelength:**240nm **HPLC/MS** **Column:** Nucleosil C18 column (250mm×2.1 mm, 5µm) **Mobile phase:**methanol-ammonium formate(6mM,pH 6.5)(80/20) **Flow rate**:150µl/min	107.
25.	Bulk drug & tablet	NIR spectrophotometry	NIRS can be used to validate the whole manufacturing process and not only as an analytical method for tablets assay.	108.
26.	Bulk drug, Capsule, Urine	Stripping voltametry	**Reagent :**phosphate buffer (pH 7.0) Adsorptive cathodic peak was observed at -1.72 V vs.Ag/AgCL. **Detection limit**:1×10^{8}M(4.5ng/ml) using 180s preconcetration.	109.

27.	Bulk drug,tablet ,capsule	Extractive spectrophotometric method	Formation of coloured chloroform extractable ion-pair complexes of the drug with bromothymol blue (BTB), bromophenol blue (BPB) and bromocresol green (BCG) in acidic medium. Absorbance maxima at 415nm **Linearity range**: 2.5–20.0, 2.5–10.0 and 2.5–12.5 mg /ml with BTB, BPB and BCG, respectively.	110.
28.	Bulk drug, sustained release tablet	HPTLC	**Mobile phase:** ethyl acetate:methanol:strong ammonia solution(80:10:10, v/v) **Detection wavelength:**238nm **Linearity range:**40-400ng	111.

TABLE 2.2 METHOD FOR ESTIMATION OF DH IN COMBINATION WITH OTHER DRUGS

SR. NO	MATRIX	OTHER ANALYTE	METHOD	DESCRIPTION	REF. NO
1.	DH	Nadolo, Nifedipin, Propranolol, Verapamil	Micellar liquid chromatography	**Column :** ODS-2 column Kromasil (150mm×4.6mm ,5µm) **Mobile phase:** 0.05M SDS–5% pentanol at pH 7 **Flow rate:** 2ml/min **Detection wavelength:**220nm **Linearity range:**2-20µg/ml. **LOD:**in the range of 0.028µg/ml for diltiazem,0.130µg/ml for verapamil **LOQ**: in the range of 0.092 to 0.431µg/ml for same compound.	112.
2.	Bulk drug , Capsule	Trazodone HCl, Famotidine	Colorimetric	Oxidation of the cited drugs with iron(III) in acidic medium. The liberated iron(II) reacts with 1,10-phenanthroline and the ferroin complex is colorimetrically measured at 510 nm **Linearity range:** 1-5,2-12,12-32µg/ml for trazodone,famotidine,diltiazem. **Molar absoprtivity:**1.06×10^5,2.9×10^4,1.2×10^4.	113.

3.	Bulk drug,tablet,human serum	Metformin,rosiglitazone,pioglitazone	RP-HPLC	**Column:**Hiber,RP-18(250mm×4.6mm) **Mobile phase:** acetonitrile:methanol:water(30:20:50 v/v) with a pH adjusted to 2.59 ± 0.02 with phosphoric acid (85%). **Flow rate:**1ml/min **Detection wavelength:**230nm	114.
4.	Capsules, pills	Clorazepate, diazepam	HPLC	**Column:** ODS-2(125×4.6mm,5µm) **Micellar mobile phase:**0.1M SDS-3%(V/V)butanol and methanol:water(70:30) **Flow rate:**0.7ml/min **Detection wavelength:**230nm **Linearity range:**2.5-20µg/ml, 4-20µg/ml, 5-40µg/ml for clorazepate,diazepam, and diltiazem respectively.	115.
5.	Bulk drug, Tablet, human serum	Pravastatin, Meloxicam, Naproxen sodium	LC-UV	**Column:**purospher star,C_{18} (250×4.6mm,5µm) **Mobile phase:**methanol:water(80:20v/v) and pH adjusted 3.4 with 85% o-phosphoric acid. **Flow rate:** 1 ml/min **Detection wavelength:**220nm	116.

RATIONALE OF SELECTING SEMI-SOLID DOSAGE FORM

DH is official in IP (2010), BP (2010), USP 31 NF 26, EP 5.0.It is marketed in India since 1995 in different dosage form like tablet, capsule, extended release tablet and capsule, injectable preparations. Number of analytical methods are available for estimation of Diltiazem Hydrochloride alone as well as in its different dosage forms. Methods are also reported for estimation of DH in complex biological samples as well as in dosage form in combination with other drugs. Novel dosage form of DH gel is recently introduced in market for treatment of anal fissures. Till date no method is reported for estimation of DH for its gel formulation. Therefore, aim of present study is to develop simple, precise, cost effective UV spectrophotometric method for estimation of DH for gel formulation. Efficacy and safety of semi-solid dosage form depends on its release profile and so it is essential to check in-vitro drug release for DH gel formulations.

4.1 LIST OF INSTRUMENTS

1. Melting point apparatus -T60 03107, EIE Instruments Pvt. Ltd. Ahmedabad.

2. UV visible double beam spectrophotometer - UV 2450 pc series, Manufactured by Shimadzu Inc. Japan.

3. High performance liquid chromatography - JASCO 200 Series, with borwin software, Jasco Inc, Japan

4. Fourier transform infrared spectrometer - JASCO FT/IR-6100 series, TGS Detector with Spectra Manager Software. Japan.

5. Analytical balance - CX-220, Citizen. Mumbai.

6. Sonicator - D-compact, EIE Instruments Pvt. Ltd. Ahmedabad.

7. Hot air oven - EIE 101, EIE instruments Pvt. Ltd. Ahmedabad.

8. Franz diffusion cell - Durasil ® Durga glass works limited. Vadodara.

9. Magnetic stirrer - BDI -143, B.D. Instrumentation. Harayana,

10. Vacuum pump - Rocker 600, Rocker. Mumabi.

11. pHmeter - PHCAL, Analab scientific instruments Pvt. Ltd. Vadodara.

12. Waterbath - EIE instruments Pvt. Ltd. Ahmedabad.

4.2 LIST OF MATERIALS

1. API Diltiazem Hydrochloride (DH) -gifted sample from TORRENT Pharmaceuticals Pvt. Ltd. Ahmedabad.

2. Potassium dihydrogen phosphate -Merck specialities Pvt Ltd. Mumbai

3. Sodium hydroxide pellets - S. D. Fine chemicals Ltd. Mumbai

4. Methanol & Acetonitrile HPLC grade- Merck specialties Pvt Ltd. Mumbai.

5. Ammonium acetate crystal - AR grade - S. D. Fine Chemicals Ltd., Mumbai

6. Triethyl amine & Glacial Acetic acid - S. D. Fine chemicals Ltd, Mumbai.

7. Dilzem gel (diltiazem hydrochloride gel 2%) - TORRENT Pharmaceuticals Ltd. Ahmedabad. Procured from local market.

8. Diltiact (diltiazem 2% gel) - manufactured by TROIKAA Pharmaceuticals Ltd., marketed by SUN Pharmaceuticals Ind. Ltd. Ahmedabad. Procured from local market.

5.1 IDENTIFICATION

5.1.1 DETERMINATION OF MELTING POINT

Melting point of API has been determined using melting point apparatus and it was compared with reported melting point as shown in Table 5.1.

Table 5.1: Melting point of pure drug.

Name of Drug	Reported Melting Point (°C) [77]	Observed Melting Point (°C)
DH	207.5 - 212	209 - 211

5.1.2 DETERMINATION OF UV SPECTRA

The UV spectra of DH (10µg/ml) in 0.1N HCl solution was measured in the range of 200-400 nm as shown in the Fig 5.1 and λ_{max} of DH was obtained at 236nm and it was matched with the λ_{max} reported in analytical profile of drug substance and excipient volume 23.

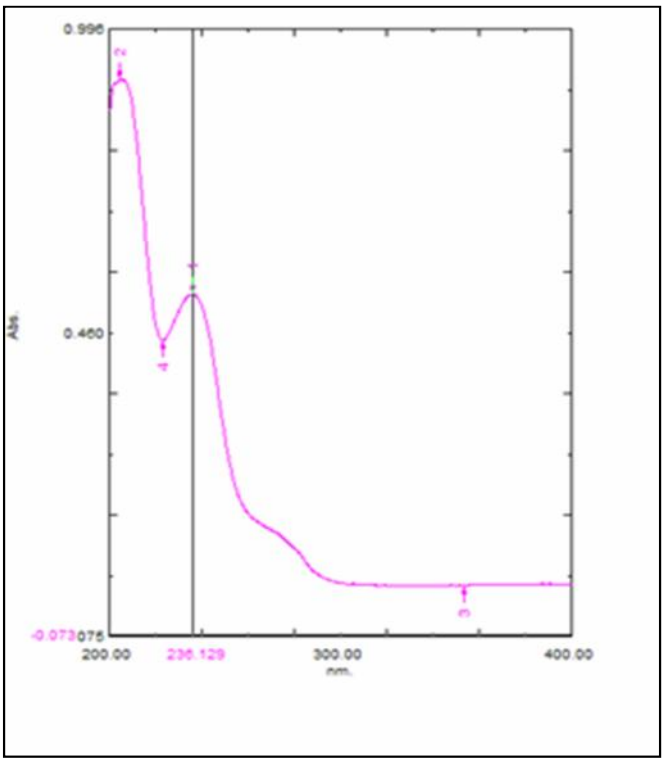

Fig.5.1: UV- Spectra of DH (10µg/ml) in 0.1 N HCl solution.

5.1.3 DETERMINATION OF FT- IR SPECTRA

FT- IR spectra of DH was taken by mixing with dried KBr and % Transmittance was measured between 400 – 4000 cm^{-1}. Peaks were overlapping with reported spectra in fingerprint region which confirms that the gifted sample is of DH.

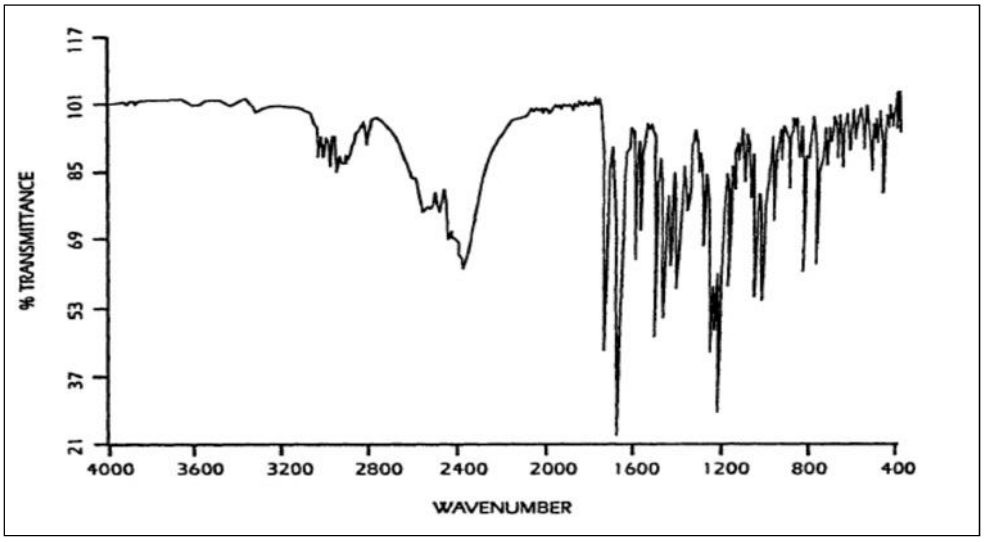

Fig.5.2 (a): Reported FT-IR spectra of DH [77]

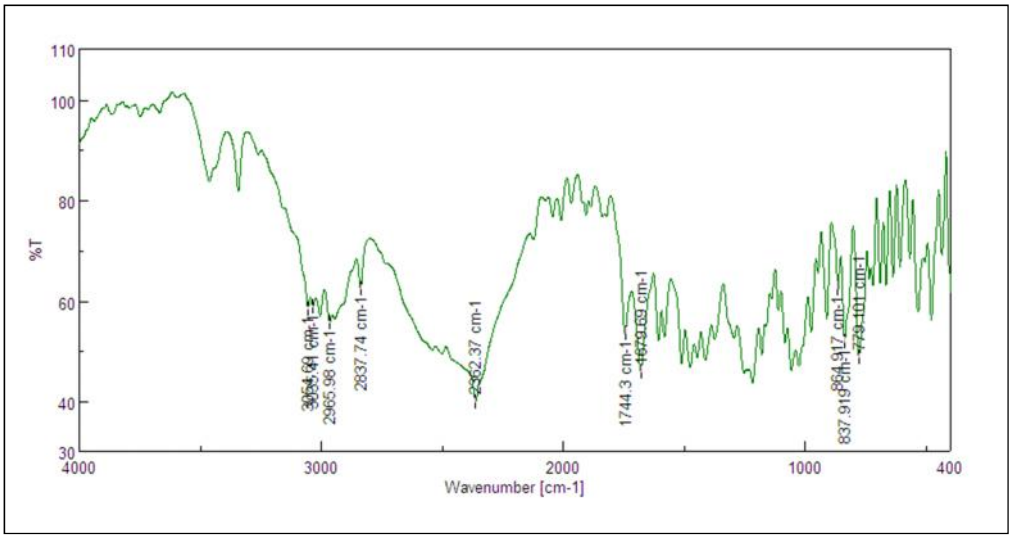

Fig.5.2 (b): Observed FT-IR spectra of DH.

5.2 DEVELOPMENT AND VALIDATION OF UV SPECTRO-PHOTOMETRIC METHOD FOR THE ESTIMATION OF DILTIAZEM HYDROCHLORIDE IN MARKETED GEL FORMULATIONS.

5.2.1 PREPARATION OF SOLUTIONS

All the solutions were protected from light throughout the experiment and prepared in amber coloured volumetric flasks.

5.2.1.1 Preparation of standard stock solution of DH

Accurately weighed 25 mg of DH was transferred to 25 ml volumetric flask and dissolved in 10 ml of D.D.W. (Double distilled water) and shake. Volume was made upto the mark with D.D.W. to obtain standard stock solution having concentration of 1000µg/ml. An aliquot of 2.5ml was further diluted to 25ml with D.D.W. to obtain working standard solution of 100µg/ml.

5.2.1.2 Preparation of sample solution

Quantity of Gel (0.25 g) equivalent to 5 mg of DH of two different marketed formulations were weighed and transferred to 50 ml volumetric flask. Individualy 25 ml of D.D.W. was added with the help of ultrasonication for 15-20min. Solution was made upto the mark with D.D.W. and filtered through whattmann filter paper no.1 to obtain sample solution of 100µg/ml.

5.2.1.3 Preparation of diluted standard solution of DH

From the standard stock solution of 100µg/ml, 1 ml was diluted to 10ml with D.D.W. to obtain standard solution of 10µg/ml. The absorbance of solution was measured at 236nm.

5.2.1.4 Preparation of sample solution for assay

From the sample solution of 100µg/ml, 1 ml was diluted to 10 ml with D.D.W. to obtain the concentration of 10µg/ml. From the absorbance at 236nm concentration was calculated from linearity equation and from that % assay was calculated.

5.2.2 METHOD VALIDATION

5.2.2.1 Linearity

Preparation of calibration curve

For calibration curve of DH, 0.25, 0.5, 1, 1.5, 2, 2.5 ml of working standard solution was accurately transferred in 10 ml volumetric flasks individualy and diluted each with D.D.W. Absorbance of all the prepared solutions were measured at 236nm.

5.2.2.2 Precision

The precision of analytical method is the degree of agreement among individual results when the method is applied to multiple sampling of homogenous samples. It provides an indication of random error in results and was expressed as coefficient of variance (CV).

5.2.2.2.1 Intraday and interday precision

Intra-day and inter-day precision was determined by measuring the absorbance of drug at three times within a day and on three different days, respectively. For intraday precision study 0.25, 1, 2.5 ml of working standard solution was transferred in each 10 ml volumetric flasks and diluted each with D.D.W. to obtain concentration of 2.5, 10, 25 µg/ml respectively. Absorbance of all the solutions were measured at 236nm.

5.2.2.2.2 Repeatability

It is a measure of precision under the same operating conditions over a short interval of time. To study the repeatability 10µg/ml solution of DH was prepared from 100µg/ml working standard solution of DH and absorbance of the solution was measured six times at 236nm.

5.2.2.3 Limit Of Detection and Limit Of Quantification(LOD/LOQ)

The LOD & LOQ were measured by using mathematical equations given below:

$$LOD = 3.3 \times \sigma / S$$

$$LOQ = 10 \times \sigma / S$$

Where,

σ = Standard deviation of intercept

S = Slope of calibration curve

5.2.2.4 Accuracy

Accuracy was determined in terms of % recovery. Recovery study was done by standard addition method. To study the accuracy, one marketed formulation of DH gel (DILTIACT gel) was taken. Quantity of gel (0.25 g) equivalent to 5 mg of DH was weighed and transferred to 50 ml volumetric flasks. Dissolved in 25 ml of D.D.W. and ultrasonicate for 20 min. Solution was made upto the mark with D.D.W. to obtain sample solution concentration of 100µg/ml.

For accuracy studies, individualy 1 ml was taken from 100µg/ml sample solution in three different 10 ml volumetric flasks and 0.8, 1, 1.2ml of working standard solution of DH was added individualy and diluted to 10ml with D.D.W. to obtain concentration of 18, 20, 22µg/ml. Absorbance of the solutions were measured at 236nm.

5.3 IN–VITRO DRUG RELEASE PROFILE FOR DILTIAZEM HYDROCHLORIDE GEL BY FRANZ DIFFUSION CELL

5.3.1 PREPARATION OF SOLUTIONS

5.3.1.1 Preparation of 0.2 M Sodium Hydroxide

0.8 g of Sodium Hydroxide was weighed and transferred to 100ml volumetric flask. Dissolved with D.D.W. and volume was made upto the mark with D.D.W. to obtain 0.2 M NaOH.

5.3.1.2 Preparation of 0.2 M Potassium Dihydrogen Phosphate

6.8 g of Potassium Dihydrogen Phosphate was weighed and transferred to 250ml volumetric flask. Dissolved with D.D.W. and volume was made upto the mark with same to obtain 0.2 M Potassium Dihydrogen Phosphate.

5.3.1.3 Preparation of Phosphate Buffer pH 7.4

50ml of 0.2 M potassium Dihydrogen Phosphate was added in 200ml volumetric flask and 39.1ml of 0.2 M sodium hydroxide was added and volume was made upto mark with D.D.W. to obtain Phosphate Buffer of pH 7.4

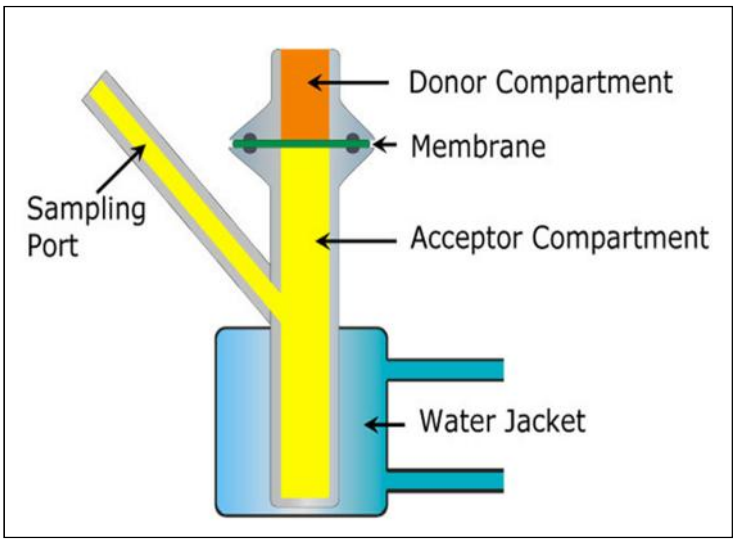

Fig.5.3: Franz diffusion cell.

5.3.2 Drug release procedure

Drug release studies were carried out using Franz diffusion cell as shown in Fig.5.4. Franz diffusion cell having internal diameter of 3.5 cm was used. Cellophane membrane was used as a model membrane. Cellophane membrane strip of 3.5×3.5 cm was cut and it was soaked in phosphate buffer pH 7.4 for 24 hr. The strip was dried and 0.025 g gel equivalent to 500µg of DH was applied on strip.

The acceptor compartment was filled with 20ml of Phosphate Buffer pH 7.4 which was maintained at 37^0C ±2 ^{0}C & the hydrodynamics were maintained by stirring with a magnetic bead at 100rpm.

Cellophane strip was in intimate contact with the Phosphate Buffer.1ml sample was withdrawn at 15, 60, 120min time interval. Volume of acceptor compartment was maintained using fresh solution. %Drug release was determined by measuring the absorbance in UV Visible spectrophotometer at 236nm.

5.4 ESTIMATION OF DILTIAZEM HYDROCHLORIDE FROM DIFFERENT MARKETED GEL FORMULATIONS USING REPORTED RP-HPLC METHOD AND TO STUDY HYDROLYTIC DEGRADATION

5.4.1 PREPARATION OF SOLUTIONS

All the solutions were protected from light throughout the study and prepared in amber color glass wares. Final dilution of all the solutions were carried out using Methanol as diluent.

5.4.1.1 Preparation of standard solution

Accurately weighed 25 mg of DH was transferred to 25 ml of volumetric flask. Dissolved in 10 ml of Mili Q water. Volume was made upto the mark with Mili Q water to obtain standard stock solution having concentration of 1000µg/ml. From this 0.5ml solution was diluted to 25ml with the diluent to obtain the final concentration of 20µg/ml.

5.4.1.2 Preparation of sample solution

Quantity of Gel (0.25 g) equivalent to 5 mg of DH, each of two marketed formulations were weighed and transferred to 50 ml volumetric flask. Individualy 25 ml of Mili Q water was used to dissolve the gel with the help of ultrasonication for 15-20min.Solution was made upto the mark with Mili Q water and filtered through whattmann filter paper no.1 to obtain sample concentration of 100µg/ml. First 2 ml solution was discarded and 2ml each of the filtrate was taken in 10ml volumetric flask and each was made upto the mark with diluent to obtain sample concentration of 20µg/ml.

5.4.1.3 Chromatographic conditions

Table 5.2: Chromatographic conditions

Column	C_{18} XTerra RP-18 (150mm×4.6mm,5µm)
Mobile phase	Methanol : Acetonitrile : Ammonium acetate buffer (38:26:36%v/v/v) +0.08%TEA pH adjusted to 7.5
Flow rate	1ml/min
Volume of injection	20µl
Detector	PDA
Detection wavelength	249nm
Retention time	4.9min
Run time	7.0min

5.4.1.4 System suitability

For system suitability parameters, five replicates of standard preparation were injected and chromatograms were recorded and following parameters were calculated as given below.

- Theoretical plate for peak in five replicate standard injection. : NLT 2500
- %RSD of peak area for five replicate standard injection : NMT 2.0
- Tailing factor of peak in five replicate standard injection : NMT 2.0
- %RSD of retention time of peak in five replicate standard injection : NMT 2.0

If system suitability passes then inject sample preparation in duplicate.

5.4.1.5 Linearity

For the calibration curve standard stock solution of 1000µg/ml was prepared as per the section 5.4.1.1 and from that 0.5ml solution was diluted to 50ml with diluent to obtain the concentration of 100µg/ml. From this solution 0.5, 1, 1.5, 2, 3ml were taken individualy and diluted to 10ml with the diluent to obtain the concentration of 5, 10, 15, 20, 30µg/ml respectively.

5.4.1.6 Sample preparation for solution stability

Quantity of Gel (0.25 g DILTIACT gel) equivalent to 5 mg of DH was weighed and transferred to 50 ml volumetric flask. Individualy 25 ml of Mili Q water was used to dissolve the gel with the help of ultrasonication for 15-20min.Solution was made upto the mark with Mili Q water and filtered through whattmann filter paper no.1 to obtain stock concentration of 100µg/ml. 2 ml of the solution was discarded. From the filtrate 2ml of the solution was taken and diluted to 10ml with diluent to obtain concentration of 20µg/ml. This solution was kept at benchtop for 24 & 48 hr and injected to the system.

5.4.2 HYDROLYTIC DEGRADATION STUDY FOR DH STANDARD AND MARKETED GEL FORMULATIONS

5.4.2.1 Preparation of DH standard solution for hydrolytic degradation

Accurately weighed 25mg of DH was transferred to RBF. Dissolved in 50ml of Mili Q water to obtain standard concentration of 500µg/ml. Solution was subjected to degradation by refluxing the solution in round bottom flask at 50^0C for 5hr and at 80^0C for 8 hrs. An aliquot of sample was withdrawn at 0, 5 & 8 hr time interval. This was diluted with the diluent and analysed as described in section 5.4.1.3.

5.4.2.2 Preparation of sample solution for hydrolytic degradation

1.25 g of gel (DILZEM gel) equivalent to 25mg of DH was weighed and transferred to 50ml volumetric flask. Dissolved in 50ml of Mili Q water with the help of ultrasonication for 15-20 min. This solution having concentration of 500µg/ml was subjected to degradation by refluxing in round bottom flask at 50^0C for 5 hr and 80^0C for 8 hrs. An aliquot of 0.4 ml was withdrawn at 0 & 8hr time interval. This was diluted upto 10 ml with the diluent and analysed as described in section 5.4.1.3.

5.4.2.3 Preparation of DH standard solution for acidic and alkaline degradation
Accurately weighed 0.5 g of DH was transferred to two 25ml volumetric flask.20ml of Mili Q water was added. Individualy pH of the solution was adjusted to 2 with 0.1M HCl and 9 with 0.1 M NaOH. Final volume was made upto 25ml with Mili Q water to obtain the conc. of 20mg/ml.

1ml solution was transferred to 25ml volumetric flask and made upto the mark with Mili Q water to obtain conc. of 800µg/ml. From this solution 0.25ml solution was diluted to 10 ml with diluent to obtain conc.of 20µg/ml.

6.1 DEVELOPMENT AND VALIDATION OF UV SPECTRO PHOTOMETRIC METHOD FOR THE ESTIMATION OF DILTIAZEM HYDROCHLORIDE IN DIFFERENT MARKETED GEL FORMULATIONS.

6.1.1 Optimization of experimental conditions

(1) Selection of solvent

- Solvent selection is very important for release % drug from matrix of gel. If proper solvent is not selected then drug will not release from gel and it will show false absorbance.
- As Diltiazem Hydrochloride is freely soluble in Chloroform & Methanol, both solvents were tried individualy. But Chloroform and in different mixture of Chloroform & Methanol reproducible results were not obtained.
- In Methanol it showed reproducible results, but along with that double distilled water was also tried & it also showed reproducible results for API and gel formulation. (Fig. 6.1 and 6.2) So to avoid wastage of Methanol, double distilled water was selected as solvent.

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SUMMARY

Simple UV spectrophotometric method was developed for DH at wavelength 236nm (λ_{max} of DH) in solvent (double distilled water). Linearity range was observed in the concentration range of 2.5-25µg/ml with the linearity equation 0.051x + 0.047 with correlation coefficient 0.998. The method is validated for specificity, accuracy, precision, repeatability as per ICH guidelines Q2 (R1). The limit of detection and quantitation were found to be 0.0589 and 0.1785µg/ml.

It is essential to check the efficacy of semi-solid dosage form by checking their release pattern and it was performed by franz diffusion cell. For in-vitro drug release 82.33% drug release was found for DILZEM gel and 79.83% for DILTIACT gel.

It is necessary to compare the developed UV spectrophotometric method with the HPLC method. So assay of gel formulations were performed by slight modification in reported RP-HPLC method, by using XTerra C_{18}(150mm×4.6mm,5µm) column with Methanol: Acetonitrile:Ammonium acetate buffer +0.08% TEA pH adjusted to 7.5 (38:26:36) as a mobile phase. The flow rate was maintained 1ml/min with injection volume 20µl.

Linearity was taken in the range of 2-30µg/ml with linearity equation 57449x – 222.8 with correlation co-efficient of 0.999.

Assay of two marketed formulations by RP-HPLC was found to be 86.86% for DILZEM gel and 87.43% for DILTIACT gel. In assay of gel formulations, HPLC chromatogram showed additional peak at retention time 4.9 whereas DH showed peak at retention time of 4.2. This peak may be due to presence of excipient, degradation of drug or other impurity component. So degradation study was performed for DH API and gel formulations in acidic, basic, neutral condition at different time and temperature. DILZEM gel showed more degradation then DILTIACT gel. From the RP-HPLC chromatogram it was observed that 14.69% degradation in DILZEM gel and 5.53% for DILTIACT gel. In acidic and alkaline condition API showed 1.80% and 6.22% degradation.

CONCLUSION

Hence, developed UV - Spectrophotometric method can't be used for the estimation of DH in gel formulations as degradation product can't be detected in UV- spectrophotometric method and it shows good %assay of DH. While in RP-HPLC method it shows less assay due to the degradation. In assay the PDA spectra of degradation product and DH was observed to be same which indicates that they have same absorptivity value and structure similarity between degradation product and DH. In literature review it was observed that DH undergoes hydrolysis and produces its major degradation product as des-acetyl diltiazem. So this degradartion product may be des-acetyl diltiazem which needs to be further analysed by LC/MS. So HPLC method is more preferrable than UV- spectrophotometric method.