"DEVELOPMENT AND VALIDATION OF UV-SPECTROPHOTOMETRIC, SPECTROFLUORIMETRIC METHODS FOR SIMULTANEOUS ESTIMATION OF MEMANTINE HYDROCHLORIDE AND DONEPEZIL HYDROCHLORIDE USING OPA-β - MERCAPTOETHANOL DERIVATIZATION IN TABLET DOSAGE FORM AND HUMAN PLASMA"

A Thesis Submitted to

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in Partial Fulfillment for the Award of Degree of

MASTER OF PHARMACY
IN
PHARMACEUTICAL ANALYSIS

BY

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May 2012

CERTIFICATE

This is to certify that the dissertation work entitled, "Development and Validation of UV-Spectrophotometric, Spectrofluorimetric methods for simultaneous estimation of Memantine Hydrochloride and Donepezil Hydrochloride using OPA - β mercaptoethanol Derivatization in tablet dosage form and human plasma" submitted by ***Mr. AJAY SINGH*** *with reg. no.* ***10MPH301*** *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, "Development and Validation of UV-Spectrophotometric, Spectrofluorimetric methods for simultaneous estimation of Memantine Hydrochloride and Donepezil Hydrochloride using OPA- β mercaptoethanol Derivatization" is based on the original work carried out by me under the guidance of Dr. Charmy S. Kothari , Assistant Professor and Mr. Nrupesh R. Patel, Assistant Professor, 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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ABBREVIATIONS

Chemicals

MEM	Memantine Hydrochloride
DH	Donepezil Hydrochloride
OPA	Ortho-pthaladehyde
BTB	Bromo-thymol blue
BCG	Bromo-cresol green
F.C.	Folin – ciocalateau reagent
NQS	1,2-Naphthoquinone-4-sulphonate
BPB	Bromo-phenol blue
FMOC	9- fluorenylmethyl chloroformate

Symbols

R^2	Correlation coefficient
°C	Degree centigrade
g	gram
<	Less than
L	Liter
L/h	Liter per hour
µg	Microgram
µL	Microliter
mL	Mililiter
mm	Milimeter
mM	Milimolar
mg	Milligram
MPa	MilliPascal
min	Minutes
ng	Nanogram
Pg.No.	Page number
pKa	Partition coefficient
%	Percentage
Ref.No.	Reference number
Sr.No.	Serial number
v/v	Volume/Volume
w/v	Weight / Volume

Others

AD	Alzheimer's disease
CAS No.	Chemical Abstract Service Number
FT-IR	Fourier Transform Infrared spectrometry
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
IP	Indian Pharmacopoeia
ICH	International Conference on Harmonization
IUPAC	International Union of Pure and Applied Chemistry
LOD	Limit of Detection
LOQ	Limit of Quantification
LC	Liquid Chromatography
LDL	Low density lipoproteins
MS	Mass Spectrometry
RSD	Relative Standard Deviation
SD	Standard Deviation
UV	Ultra violate
UV/Vis	Ultra violate/Visible
USP	United States Pharmacopoeia

ABSTRACT

This paper describes validated UV spectrophotometric, spectrofluorimetric methods for the simultaneous estimation of memantine hydrochloride (MEM) and donepezil hydrochloride (DH) in bulk and combined tablet dosage form and spectrofluorimetric method for estimation of MEM and DH in human plasma. The proposed methods include derivatization of MEM with OPA-βmercaptoethanol due to lack of chromophoric group. UV Spectrophotometric method includes simultaneous equation method that involves measurement of absorbance at two wavelengths i.e. at 337 nm (λ_{max} of MEM) and 312.6 nm (λ_{max} of DH) in methanol. Linearity range was observed in the concentration range 5-80 µg/mL with R^2 0.9984 and 0.9987, respectively for MEM and DH. LOD and LOQ were found to be 1.220 & 3.698 µg/mL for MEM respectively and 0.775 & 2.347 µg/mL for DH respectively. Spectrofluorimetric method includes synchronous spectrum method using delta value 65 (low sensitivity mode) that involves measurement of fluorescence intensity at two wavelengths in synchronous spectra of drugs i.e. at 450 nm for MEM and 361 nm for DH in borate buffer. Linearity range was observed in the concentration range 1-30 µg/mL for both the drugs with R^2 0.9986 and 0.9983, respectively for MEM and DH. LOD and LOQ were found to be 0.228 & 0.691 µg/mL for MEM, respectively and 0.135 & 0.410 µg/mL for DH, respectively. For estimation of MEM & DH in human plasma extraction was carried out using 5% TCA for protein precipitation followed by liquid-liquid extraction with 5% IPA in n-Hexane. Under spectrofluorimetric conditions delta value 65 applied in synchronous mode (medium sensitivity mode) and wavelength used in analysis were 389 nm and 420 nm respectively for MEM and DH. Linearity range was observed in the concentration range 5-105 ng/mL and 50-300 ng/mL for DH and MEM with R^2 0.9965 and 0.9951, respectively. Percentage recovery was found to be 74.43%-83.33% and 77.85%-83.68%, for MEM and DH, respectively.

Keywords – Human plasma, Donepezil hydrochloride, Memantine hydrochloride, Synchronous spectrofluorimetry, UV Spectrophotometry, Tablet dosage form.

Chapter 1
Introduction

1.1 INTRODUCTION OF DISEASE

Alzheimer's disease is a progressive neurologic disease of the brain leading to the irreversible loss of neurons and the loss of intellectual abilities, including memory and reasoning, which become severe enough to impede social or occupational functioning. Alzheimer's disease is also known as simply Alzheimer's, and Senile Dementia of the Alzheimer Type (SDAT).

During the course of the disease plaques and tangles develop within the structure of the brain. This causes brain cells to die. Patients with Alzheimer's also have a deficiency in the levels of some vital brain chemicals which are involved with the transmission of messages in the brain – neurotransmitters[1].

Oysius Alzheimer was a German neuropathologist and psychiatrist. He is credited with identifying the first published case of "presenile dementia" in 1906, which Kraepelin later identified as Alzheimer's disease - naming it after his colleague.

Loss of cognitive ability with age is considered to be a normal process whose rate and extent is very variable. AD was originally defined as presenile dementia, but it now appears that the same pathology underlies the dementia irrespective of the age of onset. AD refers to dementia that does not have an antecedent cause, such as stroke, brain trauma or alcohol. Its prevalence rises sharply with age, from about 5% at 65 to 90% or more at 95. Until recently, age-related dementia was considered to result from the steady loss of neurons that normally goes on throughout life, possibly accelerated by a failing blood supply associated with atherosclerosis. Studies over the past three decades have, however, revealed specific genetic and molecular mechanisms underlying AD, which have opened potential new therapeutic opportunities[2,3].

PATHOGENESIS OF ALZHEIMER'S DISEASE

Alzheimer's disease is associated with brain shrinkage and localised loss of neurons, mainly in the hippocampus and basal forebrain. The loss of cholinergic neurons in the hippocampus and frontal cortex is a feature of the disease, and is thought to underlie the cognitive deficit and loss of short-term memory that occur in AD. Two microscopic features are characteristic of the disease, namely extracellular *amyloid plaques*, consisting of amorphous extracellular deposits

of β-amyloid protein (known as Aβ), and intraneuronal *neurofibrillary tangles*, comprising filaments of a phosphorylated form of a microtubule-associated protein (Tau). Both of these deposits are protein aggregates that result from misfolding of native proteins, as discussed above. They appear also in normal brains, although in smaller numbers. The early appearance of amyloid deposits presages the development of AD, although symptoms may not develop for many years. Altered processing of amyloid protein from its precursor is now recognised as the key to the pathogenesis of AD. This conclusion is based on several lines of evidence, particularly the genetic analysis of certain, relatively rare, types of familial AD, in which mutations of the APP gene, or of other genes that control amyloid processing, have been discovered. The APP gene resides on chromosome 21, which is duplicated in Down's syndrome, in which early AD-like dementia occurs in association with overexpression of APP[4,5].

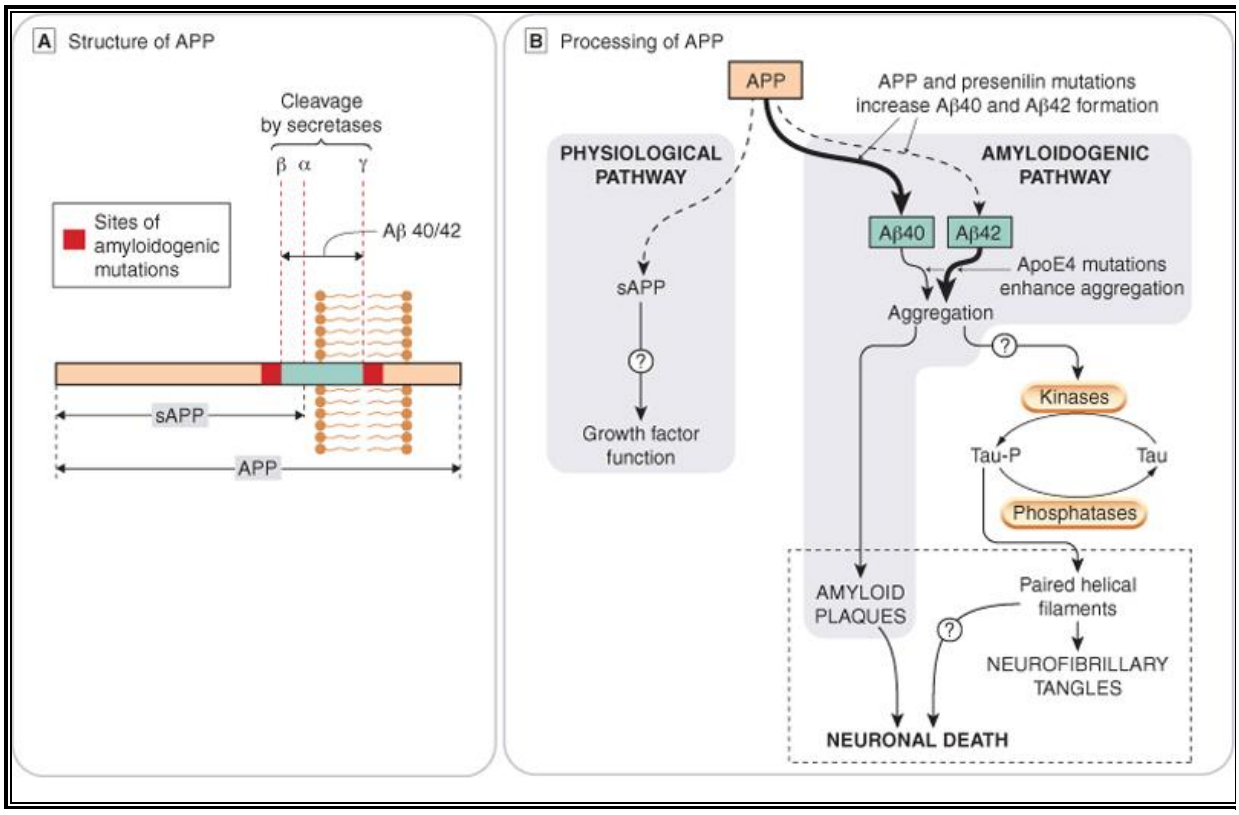

Fig.1.1: Pathogenesis of Alzheimer's disease.

Structure of amyloid precursor protein (APP), showing origin of secreted APP (sAPP) and Aβ amyloid protein. The regions involved in amyloidogenic mutations discovered in some cases of familial Alzheimer's disease are shown flanking the Aβ sequence. APP cleavage involves three proteases: secretases α, β and γ. α-Secretase produces soluble APP, whereas β- and γ-secretases generate Aβ amyloid protein. γ-Secretase can cut at different points, generating Aβ peptides of varying lengths, including Aβ40 and Aβ42, the latter having a high tendency to aggregate as amyloid plaques[6].

THERAPEUTIC APPROACHES :

Recent advances in understanding the process of neurodegeneration in AD have yet to result in therapies able to retard it. Currently, cholinesterase inhibitors and memantine are the only drugs approved for treating AD, although many drugs have been claimed to improve cognitive performance and several new approaches are being explored.

Cholinesterase inhibitors :

Tacrine, the first drug approved for treating AD, was investigated on the basis that enhancement of cholinergic transmission might compensate for the cholinergic deficit. Trials showed modest improvements in tests of memory and cognition in about 40% of AD patients, but no improvement in other functional measures that affect quality of life. Tacrine has to be given four times daily and produces cholinergic side effects such as nausea and abdominal cramps, as well as hepatotoxicity in some patients, so it is far from an ideal drug. Later compounds, which also have limited efficacy but are more effective than tacrine in improving quality of life, include donepezil, rivastigmine and galantamine.These drugs produce a measurable, although slight, improvement of cognitive function in AD patients, but this may be too small to be significant in terms of everyday life.

There is some evidence from laboratory studies that cholinesterase inhibitors may act somehow to reduce the formation or neurotoxicity of Aβ, and therefore retard the progression of AD as well as producing symptomatic benefit. Clinical trials, however, have shown only a small improvement in cognitive function, with no effect on disease progression[2].

Other drugs aimed at improving cholinergic function that are being investigated include other cholinesterase inhibitors and a variety of muscarinic and nicotinic receptor agonists, none of

which look promising on the basis of early clinical results[7].

Other drugs

Dihydroergotamuine was used for many years to treat dementia. It acts as a cerebral vasodilator, but trials showed it to produce little if any cognitive improvement. 'Nootropic' drugs such as **piracetam** and **aniracetam** improve memory in animal tests, possibly by enhancing glutamate release, but are probably ineffective in AD.

Inhibiting neurodegeneration

- For most of the disorders discussed in this chapter, including AD, the Holy Grail, which so far eludes us, would be a drug that retards neurodegeneration. Now that we have several well-characterised targets, such as Aβ formation by the β- and γ-secretases, and Aβ neurotoxicity, together with a range of transgenic animal models of AD on which compounds can be tested, the prospects certainly look brighter than they did a decade ago. Particular developments are worth mentioning
- Inhibitors of β- and γ-secretase have been identified and are undergoing c linical trials. Unfortunately, γ-secretase plays a role in other signalling pathways besides Aβ formation, so inhibitors are likely to produce unwanted as well as beneficial effects.

Currently, the only drugs approved for the treatment of AD are various cholinesterase inhibitors and memantine, an NMDA receptor antagonist believed to work by inhibiting glutamate-induced excitotoxicity. It has only mild side effects, and clinical trials data show it to produce a slight but significant improvement in cognitive function in moderate-to-severe AD[8,9].

1.2 DRUG PROFILE

1.2.1 MEANTINE HYDROCHLORIDE[10,11,12,13,14]:

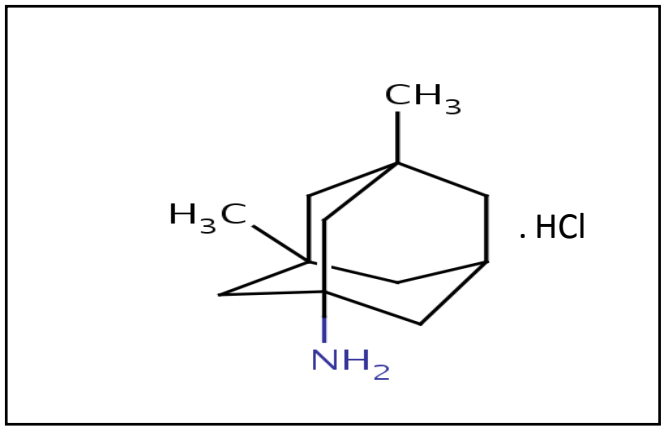

Fig.1.2: Structure of MEM

- IUPAC Name : 3,5-dimethyladamantan-1-amine Hydrochloride
- Molecular formula: C_{12} H_{21} N-HCI
- molecular mass: 215.76 (free base: 179.20)
- Appearance : white crystalline powder
- log(P): 3.28
- pKa: 10.42
- solubility of the hydrochloride salt: 3.5% in a pH 6.5 aqueous solution at 25 °C, soluble in methanol, water
- Melting point : 290-295^0 C
- Official status : USP priority monograph

- MECHANISM OF ACTION

Memantine exerts its action through uncompetitive NMDA receptor antagonism, binding preferentially to the NMDA receptor-operated cation channels.

Table 1.1: Pharmacokinetic parameters of DH

Bioavailabilty and dose proportionality	100 % bioavialable, linear PK over 5-40 mg single dose
T_{max}	4-6 hours
$T_{1/2}$	60-80 hours
Protein binding	42-65 %
Distribution	9-11 L/Kg
Metabolism and Elimaination	Little metabolism, largely (75-90%) excreted unchanged in urine
Drug- Drug interaction	Minimal interaction with drugs that are substrate of CYP450 enzymes,no adverse reaction between memantine and donepezil in-vivo
Adverse effect	Not severe, minimal effects on memory function and information processing in healthy subjects

1.2.2 DONEPEZIL HYDROCHLORIDE[10,11,16,17]

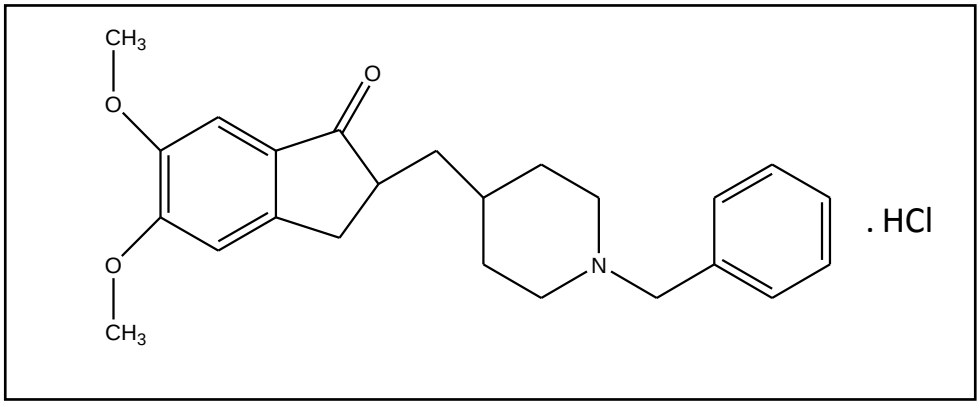

Fig. 1.3: Structure of DH

- IUPAC Name : 2-[(1-benzylpiperidin-4-yl)methyl]-5,6-dimethoxy-2,3-dihydro-1H-inden-1-one Hydrochloride
- molecular formula : $C_{24}H_{29}NO_3$. HCl
- molecular mass : 415.96 (Free Base 379.492)
- Appearance : white crystalline powder
- log(P) : 3.6
- pKa: 8.90
- solubilty : soluble in water and methanol
- Melting point : 211-212^0 C
- Official status : IP(2010)
- Mechanism of action :

It has been demonstrated that Alzheimer's disease is associated with a relative decrease in the activity of the cholinergic system in the cerebral cortex and other areas of the brain. Studies suggest that donepezil hydrochloride exerts its therapeutic effect by enhancing cholinergic

function in the central nervous system. This is accomplished by increasing the concentration of acetylcholine through reversible inhibition of acetylcholinesterase. Donepezil hydrochloride is a specific and reversible inhibitor of acetylcholinesterase, the predominant cholinesterase in the brain. Donepezil hydrochloride was found in vitro to be over 1000 times more potent an inhibitor of this enzyme than of butyrylcholinesterase, an enzyme which is present mainly outside the central nervous system.

Table1.2: Pharmacokinetic parameters of MEM

Bioavailabilty and dose proportionality	100 % bioavailable, produces highly predictable plasma concentrations with plasma concentrations and area under the curve rise in proportion to the dose
T_{max}	3- 4 Hrs
$T_{1/2}$	70 Hrs
Protein binding	96 %
Distribution	12 L/Kg
Metabolism and Elimination	4 metabolites , 2 are active
Drug- Drug interaction	Didn't affect frusemide, digoxin, and warfarin protein binding
Adverse effect	inseverity and transient in nature, diarrhoea, muscle cramps, fatigue, nausea, vomiting and insomnia

1.3 RATIONAL OF COMBINATION

Memantine, a selective *N*-methyl-D-aspartic acid receptor antagonist, and cholinesterase inhibitors such as donepezil have demonstrated efficacy in clinical trials. Preclinical evidence suggests that the 2 drugs may act synergistically to produce a significantly greater increase in acetylcholine to that seen with donepezil treatment alone, because of their different modes of action.

Patients with moderate to severe Alzheimer's disease (AD) showed significantly greater improvement in cognition, function, behaviour, and global outcome after combination treatment with memantine and donepezil versus placebo plus donepezil treatment[2,6].

1.4 INTRODUCTION TO DERIVATIZATION REACTION

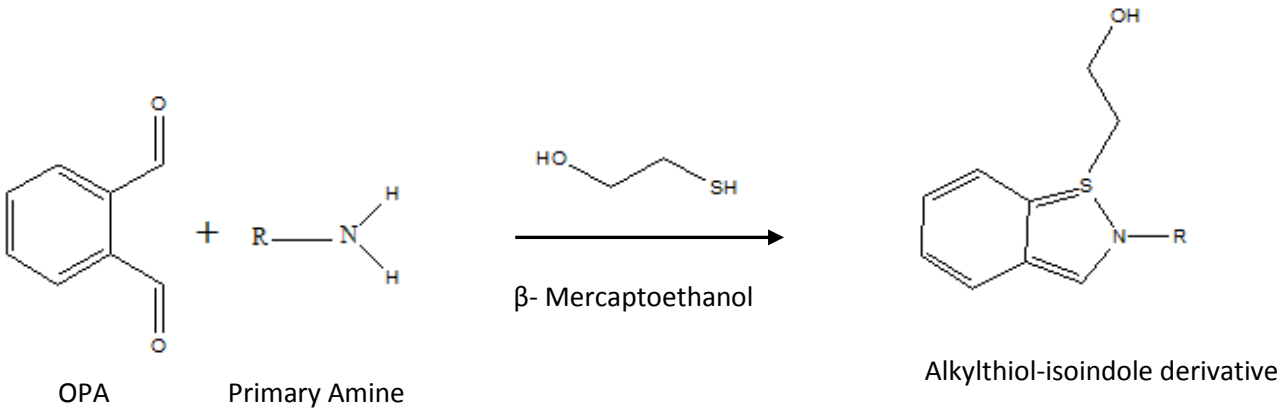

Fig.1.4: Derivatization Reaction

OPA reacts with primary amines in presence of any sulphur containing compound like β-mercaptoethanol and gives alkylthiol- isoindole derivative under basic pH condition (above 9.0) which shows λ_{max} near 337 – 340 nm and have fluroscent property of λexci near 340 nm and λemi near 455 nm.

Product of this drivatization process is more stable as decrease in amount of aqueous phase in reaction condition[17].

1.5 INTRODUCTION TO METHOD OF ANALYSIS AND METHOD VALIDATION

UV- VIS SPETROPHOTOMETRIC METHOD FOR ANALYSIS OF DRUG COMPONENTS

SPECTROFLUORIMETRIC METHOD FOR ANALYSIS OF DRUG COMPONENTS

BIOANALYTICAL METHOD DEVELOPMENT

VALIDATION OF ANALYTICAL METHODS

VALIDATION OF BIOANALYTICAL METHOD VALIDATION

AIM & OBJECTIVE

Pharmaceutical products formulated with more than one drug, typically referred to as combination products, are intended to meet previously unmet patients need by combining the therapeutic effects of two or more drugs in one product. These combination products can present daunting challenges to the analytical chemist responsible for the development and validation of analytical methods.

Development and validation of analytical method (Spectrophotometric, High performance liquid chromatography (HPLC), & High performance thin layer chromatography (HPTLC)) is carried out for drug products containing more than one active ingredient.

Basic criteria for new method development of drug analysis:

- The drug or drug combination may not be official in any pharmacopoeias.
- A proper analytical procedure for the drug may not be available in the literature due to patent regulations,
- Analytical methods may not be available for the drug in the form of a formulation due to the interference caused by the formulation excipients,
- Analytical methods for the quantification of the drug in biological fluids may not be available,
- Analytical methods for a drug in combination with other drugs may not be available,

- The existing analytical procedures may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedures and these may not be reliable[18].

1.5.1 UV-VIS Spectrophotometric Methods of Analysis for Drugs in Combination

Ultraviolet-visible spectroscopy or ultraviolet –visible spectroscopy (UV-Vis) involves the spectroscopy of photons in the UV-visible region. This means it uses light in the visible and adjacent (near ultraviolet (UV) and near infrared (NIR) ranges. The absorption in the visible ranges directly affect the color of the chemicals involved. In the region of the electromagnetic spectrum, molecules undergo electronic transitions .this technique is complementary to fluorescence spectroscopy ,in that fluorescence deals with transitions from the excited state to the ground state ,while absorption measures transitions from the ground state to the excited state .

The spectrophotometric assay of drugs rarely involves the measurement of absorbance of samples containing only one absorbing component. The pharmaceutical analyst frequently encounters the situation where the concentration of one or more substances is required in samples known to contain other absorbing substances, which potentially interfere in the assay. If the formula of the samples is known, the identity and concentration of the interfering substance are known and the extent of interference in the assay may be determined.

A number of modifications to the simple spectrophotometric procedure are available to the analyst, which may eliminate certain sources of interference and permit the accurate determination of all of the absorbing components. Each modification of the basic procedure may be applied if certain criteria are satisfied.

The basis of all the spectrophotometric techniques for multicomponent samples is the property that at all wavelengths:

- the absorbance of a solution is the sum of absorbance of the individual components or
- The measured absorbance is the difference between the total absorbance of the solution in the sample cell and that of the solution in the reference cell.

There are various spectrophotometric methods are available which can be used for the analysis of a combination samples. Following methods can be used

- Simultaneous equation method
- Derivative spectrophotometric method
- Absorbance ratio method (Q-Absorbance method)
- Difference Spectrophotometry
- Absorbance correction method
- Geometric correction method
- Orthogonal polynomial method
- Least square approximation
- Dual wavelength spectrometry
- Assay as a single –component sample
- Assay using absorbance corrected for interference

Simultaneous Equation Method

If a sample contains two absorbing drugs (X and Y) each of which absorbs at the λ_{max} of the other (as shown in figure 1.1 λ_1 and& λ_2), it may be possible to determine both drugs by the technique of simultaneous equation (Vierodt's method) provided that certain criteria apply.

The information's required are:

- the absorptivities of X at λ_1 and λ_2, a_{x1} and a_{x2} respectively
- the absorptivities of Y at λ_1 andλ_2, a_{y1} and a_{y2} respectively
- The absorbance of the diluted sample at λ_1 and λ_2, A_1 and A_2 respectively.

Let Cx and Cy be the concentration of X and Y respectively in the diluted samples.

Two equations are constructed based upon the fact that at λ_1 and λ_2, the absorbance of the mixture is the sum of the individual absorbance of X and Y.

At λ_1

$A_1 = a_{x1}bC_x + a_{y1}bC_y$ ..... (1)

At λ_2

$A_2 = a_{x2}bC_x + a_{y2}bC_y$ ..... (2)

For measurements in 1 cm cells, b =1.

Rearrange equation (2)

$$Cy = \frac{(A2 - ax2 \,.\, Cx)}{ay2}$$

Substituting for C_y in eq. (1) and rearranging gives

$C_x = (A_2\, a_{y1} - A_1\, a_{y2}) / (ax_2\, a_{y1} - a_{x1}\, a_{y2})$

$C_y = (A_1\, a_{x2} - A_2\, a_{x1}) / (ax_2\, a_{y1} - a_{x1}\, a_{y2})$

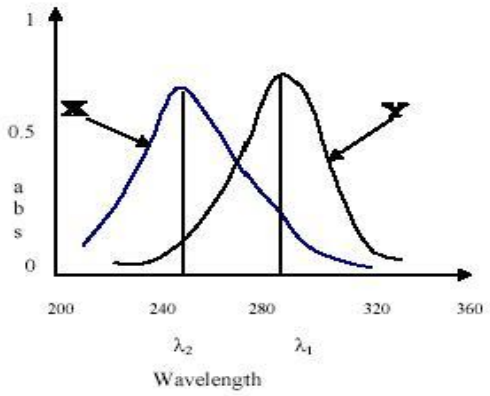

Fig.1.5: The overlain spectra of substance X and Y, showing the wavelength for the assay of X and Y in admixture by the method of simultaneous equation.

Criteria for obtaining maximum precision have been suggested by Glenn. According to him absorbance ratio place limits on the relative concentrations of the components of the mixture.

(A_2/A_1) / (a_{x2}/a_{x1}) and (a_{y2}/ay_1)/ $(A2/A_1)$

The criteria are that the ratios should lie outside the range 0.1- 2.0 for the precise determination of Y and X respectively. These criteria are satisfied only when the λmax of the two components are reasonably dissimilar. An additional criterion is that the two components do not interact chemically, thereby negating the initial assumption that the total absorbance is the sum of the individual absorbance. The additive of the absorbance should always be confirmed in the development of a new application of this technique[18,19].

1.5.2 Introduction to Spectrofluorimetric Methods of Analysis for Drugs in Combination

Fluorescence is a spectrochemical method of analysis where the molecules of the analyte are excited by irradiation at a certain wavelength and emit radiation of a different wavelength. The emission spectrum provides information for both qualitative and quantitative analysis.

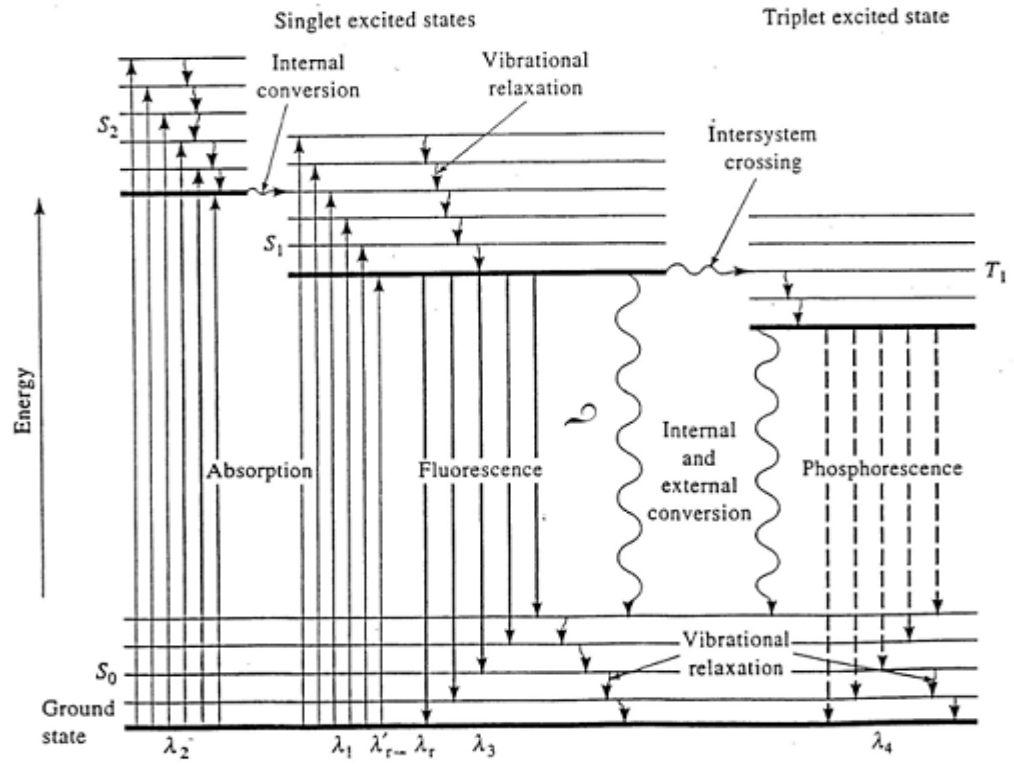

Fig. 1.6 : Electronic transition energy level diagram

As shown in Figure, when light of an appropriate wavelength is absorbed by a molecule (i.e., excitation) , the electronic state of the molecule changes from the ground state to one of many vibrational levels in one of the excited electronic states. The excited electronic state is usually the first excited singlet state, S1 (Figure 1). Once the molecule is in this excited state, relaxation can occur via several processes, Fluorescence is one of these processes and results in the emission of light.

Following absorption, a number of vibrational levels of th e excited state are populated. Molecules in these higher vibrational levels then relax to the lowest vibrational level of the

excited state (vibrational relaxation). From the lowest vibrational level, several processes can cause the molecule to relax to its ground state. The most important pathways are:

1. Collisional deactivation (external conversion) leading to non radiative relaxation.

2. Intersystem Crossing (10-9 s): In this process, if the energy states of the singlet state overlaps those of the triplet state, as illustrated in Figure 1, vibrational coupling can occur between the two states. Molecules in the s ingle excited state can cross over to the triplet excited state.

3. Phosphorescence: This is the relaxation of the molecule from the triplet excited state to the singlet ground stat e with emission of light. Because this is a classically forbidden transition, the triplet state has a long lifetime and the rate of phosphorescence is slow (10-2 to 100 sec).

4. Fluorescence: Corresponds to the relaxation of the molecule from the singlet excited state to the singlet ground state with emission of light. Fluorescence has short life tim e (~10^{-8} sec) so that in many molecules it can compete favorably with collisional deactivation, intersystem crossing and phosphorescence. The wavelength (and thus the energy) of the light emitted is dependent on the energy gap between the ground state and the singlet excited state. An overall energy balance for the fluorescence process could be written as:

$$E_{fluor} = E_{abs} - E_{vib} - E_{solv.relax}$$

Where E_{fluor} is the energy of the emitted light, E_{abs} is the energy of the light absorbed by the molecule during excitation, and E_{vib} is the energy lost by the molecule from vibrational relaxation and $E_{solv.relax}$ term arises from the need for the solvent cage of the molecule to reorient itself in the excited state and then again when the molecule relaxes to the ground state. As can be seen from Equation , fluorescence energy is always less than the absorption energy for a given molecule. Thus the emitted light is observed at longer wavelengths than the excitation.

5. Internal Conversion: Direct vibrational coupling between the ground and excited electronic states (vibronic level overlap) and quantum mechanical tunneling (no direct vibronic overlap but small energy gap) are internal conversion processes. This is a rapid process ($10^{-1\ 2}$ sec)

relative to the average lifetime of the lowest excited singlet state (10^{-8} sec) and therefore competes effectively with fluorescence in most molecules.

Other processes, which may compete with fluorescence, are excited state isomerization, photoionization, photodissociation and acid-base equilibria. Fluorescence intensity may also be reduced or eliminated if the luminescing molecule forms ground or excited state complexes (quenching)[19].

SYNCHRONOUS SPECTROFLUROMETRIC ESTIMATION

Excitation-emission (EE) spectrofluorimetry is an analytical technique of moderate selectivity and extremely high sensitivity, which can be applied to the detection of a very wide range of analytes in environmental and biological samples. Its capacity of detection is approximately one order of magnitude greater than that of molecular absorption spectroscopy and its selectivity is also clearly greater than that of other spectroscopic methods , so it is applied for the analysis of concentrations in the ng/mL range and, sometimes, in the pg/mL range in complex matrices. However, these performances in multi-component analysis fall off considerably due to the overlapping of the spectra, so that resolution of mixtures is unsatisfactory. The limited selectivity of EE spectrofluorimetry can be improved by applying total luminescence or synchronous scanning fluorescence techniques. Synchronous fluorescence (SF) was introduced in the 1980s and is possible in the following modes:

Constant-wavelength synchronous luminescence (CWSL):

This method involves the registration of the fluorescence spectra while simultaneously scanning for the excitation and emis-sion wavelengths at the same velocity (synchronously) and consequently keeping a constant difference or increment between the wavelength of emission and that of excitation.

Constant-energy synchronous luminescence (CESL):

In this case, the scanning is carried out keeping a constant energy difference between the excitation and the emission monochromators.

Variable-angle SF spectrometry:

This implements different scan speeds for the excitation and emission mono-chromators, allowing the slope of the straight line in the EE matrix (EEM) to vary from 1. This scanning mode offers considerable flexibility and improved performance compared to other scan modes. Cabaniss[distinguished between variable-angle scans with the slope defined in wavelength (VAW) and energy (VAE).

Both CWSL and CESL correspond to a straight line with a slope of 1 across an EEM with axes in wavelength and energy units, respectively.

Although SF is a very well-known, well-established technique, for decades, a lot of limitations have ham-pered its application to environmental, food, industrial and biological samples [e.g., inner-filter effect (especially secondary inner filter), quenching, Raman and Rayleigh scattering (although lower than in EE fluorimetry), background signals, and strong overlap of fluorescent signals from multi-component mixtures].

However, the appearance of modern pre-treatment procedures (i.e. immunoaffinity purification or enzy-matic hydrolysis) and pre-concentration procedures (generally on solid sorbents), or development of direct fluorimetry measurements on solid surface have facilitated the application of SF to complex matrices (including vegetables, foods and fuels). In addition to this, weakly-fluorescent or non-fluorescent compounds can be converted into fluorescent species (improving their quantum fluorescence yield) by means of a reagent, hydrolysis, thermal treatment, the solvent-polarity effect, complexation reactions, or advanced fluorogenic labeling, thereby spreading applications of this technique. Recently, derivatization reactions were also frequently applied to obtaining thermodynamic parameters and reaction mechanisms by spectrofluorimetry.

In addition to this, the deeper knowledge reported every year about micellar media and its application to fluorimetry have allowed significant lowering of the limits of detection (LODs) of the target analytes determined by SF.

As regards instrumental and technical improvements, incorporation of new narrow-banded excitation sources (e.g., dye laser) and the rapid cooling of samples (at temperatures as low as 77 K) makes it possible to get narrower emission lines from isolated target molecules trapped in

the matrix during cooling, so facilitating the resolution of complex mixtures. Besides this, the simultaneous application of magnetic field and polari-zation to resonant SF spectrometry (i.e. for Dk= 0 nm) is another popular innovative approach to eliminating the background scattered light observed in fluorescence. In addition to this, total internal reflection fluorescence (TIRF) is a hot topic in SF, due to its great specificity in the study of interfacial properties. Finally, SF has recently been incorporated into sequential-injection analysis (SIA) as detector for accurate determination of a lot of fluorescent compounds in an easy, automated way.

Among the principal advantages of SF are improved selectivity with respect to EE spectrofluorimetry, higher sensitivity, decreased light-scattering interference, speed, simplicity of spectral complexity, and very low costs. SF provides results in good agreement with those from high performance liquid chromatography (HPLC), gas chromatography with mass spectrometry (GC-MS), graphite furnace atomic absorption spectrometry (GF-AAS), and ultraviolet (UV) spectrophotometry.

Sometimes, SF provides even better results than those from GC-MS or infrared (IR) spectroscopy, al-though results and conclusions seem to depend on the matrix studied. Thus, SF has advantages over HPLC in crude-petroleum oils because heavy compounds tend to be adsorbed onto the analytical column and light compounds can be lost by evaporation. Nevertheless, HPLC is superior to SF as analytical tool in a lot of environmental and biological matrices. Among the disadvantages of SF as an analytical tool, it is worth mentioning its dependence on a lot of factors:

Polarity and viscosity of the solvent, concentration of salts; interferences, photochemical decomposition of the fluorescent compound , pH, temperature, incubation time of samples when needed, derivatization protocol when applied, extractive procedure, pre-treatment procedures order of adding reagents and overall occurrence of quenching self-absorption, fluorescence resonance energy transfer (FRET) and inner-filter effects, which frequently cause a lot of problems in quantitative determinations, especially in very concentrated and complex samples[20,21] .

1.5.3 INTRODUCTION TO BIO-ANALYTICAL METHOD

The quantitative and qualitative analysis of drugs in biological matrix, such as whole blood, plasma, serum, urine, feces, saliva, sputum and various discrete tissues, has witnessed immense attention in recent years. In particular analysis of drugs in biological matrix i.e. bio-analytical method has major role both in the development of more selective and effective drugs and in understanding their therapeutic and toxic effects. It also provides a basis for bioavailability, toxicokinetic, tissue distribution, pharmacokinetics, clinical, bioequivalence, biopharmaceutics studies and the influences of co-medication that have to be known for a new drug to be approved.

Once an appropriate drug is selected from drug discovery or drug development it is required to develop quantitative methods to determine concentration of drug and if necessary metabolites in biological matrix. These methods are used to support several activities in drug development including formulation research, GLP, toxicology, clinical pharmacology and clinical research studies. Problems related to bioavailability and bioequivalence, new drug development and discovery, drug abuse, clinical, pharmacokinetic and drug research are highly dependent on analysis methodology of drug in biological fluid. The assay is also assuming more importance in research and novel drug delivery system[22].

The objective of biological matrix assay of drugs is production of an accurate assessment of the concentration of a known drug or of a particular metabolite of known drug, in biological matrix such as blood, plasma, tissue fluid or urine. The development of bio-analytical methods for the quantitative evaluation of drugs and their metabolites in animal biological matrix is significant for the successful conduct of bioavailability, toxicokinetic and pharmacokinetics studies when it is in pre-clinical phase. Clinical trials focus in determining the process by which a drug is released from dosage form and moves to the site of action. However drug concentration usually cannot be readily measured directly at the site of action. Based on the premise that the drug at the site of action is in equilibrium with drug in blood or plasma, it is possible to obtain an indirect measure of drug response by monitoring drug levels in plasma. Thus development of bio-analytical methods for the quantitative evaluation of drugs and their metabolites in human plasma is critical for the successful conduct of bioavailability, safety,

pharmacokinetics, clinical, dose linearity, and bioequivalence and bio-pharmaceutics studies[23].

Detection of a drug or its metabolite in biological matrix is usually complicated by the matrix such as endogenous compounds and also by non-drug exogenous compounds, such compounds can interfere to detect and determine the materials of pharmacological interest.

Clearly, traditional approaches for analysis are not capable of meeting specialized needs created by improvements in drug discovery and development. Rapid, high throughput, sensitive and selective methods are now a requisite for bioanalytical. Also the ability to analyze trace mixtures, using an instrumental configuration compatible with screening approaches, emerged as an important feature. These demands underscored the importance of analytical instrumentation and the creation of novel strategies.

Various sophisticated techniques have been developed to allow the rapid separation and quantification of trace components of complex mixtures in biological matrix. In order to extract the drug from biological matrix and make it available for analysis different extraction techniques are used like precipitation, liquid-liquid and solid-phase extraction. Several methods have been applied in the analysis of drugs and their metabolites, such as radioimmunoassay (RIA), capillary electrophoresis (CE), gas chromatography (GC), GC-mass spectrometry (GC-MS), high performance liquid chromatography (HPLC) with UV, fluorescence, radioactivity and mass spectrometric detection (MS), Liquid chromatography-mass spectrometry (LC-MS) has become an ideal and widely used method in the analysis of drugs and their metabolites due to its unmatched sensitivity, extraordinary sensitivity and rapid rate of analysis.

A bio-analytical method has three core element method development, method validation and application of method to clinical pharmacology and bio-pharmaceutics studies. Method development involves the initial trials for selection of extraction procedure and setting of detection parameters for desired quantification of drug in biological matrix. Method validation is performed to demonstrate that a particular method used for quantitative measurement of drug and/or metabolite is reliable and reproducible for intended use. The validated method is applied to the study samples with known samples with predefined acceptance criteria. The obtained values are used to calculate the pharmacokinetics parameters for the anticipated end results.

Introduction to sample preparation

The aim of sample preparation is to isolate selectively the analyte of interest from the bulk of the matrix. The reason for the isolation may be to increase analyte concentration or to remove endogenous material in the sample that may be deleterious to the performance of the chromatography system, such as proteins.

Sample preparation/treatment is essential step of chromatographic analysis and intended for:

- Improve detection limit
- Improve specificity
- Improve reproducibility
- Improve recovery
- Improve instrument life

While selecting the method, following criteria should be noticed:

- Composition of matrix
- Chemical structure of analyte
- Lower limit of quantification (LLOQ)
- Specificity
- Reproducibility
- Recovery
- Time required for sample preparation[16]

The main sample preparation techniques used within bio-analysis are:

1. Solid Phase Extraction (SPE),
2. Liquid-Liquid Extraction,
3. Protein Precipitation and

Solid phase extraction (SPE)

"Selective retardation of analyte using solid sorbent under specific condition". SPE is based on the selective adsorption (absorption on surface) mechanism. If the targeted analyte are adsorbed on the solid phase, they can selectively be removed/eluted by using an appropriate elution (removal on analyte from adsorbent) solvent (solvent or solvent mixture).

Liquid-liquid extraction

"Selective extraction of intended analyte present in liquid sample through immiscible organic solvent." Liquid-liquid extraction is based on differential solubility and partitioning equilibrium, it required two immiscible phases, in most of the cases one aqueous and second organic phase, both phases must be immiscible.

Analyte can be removed from the matrix selectively be choosing a suitable extraction solvent and buffering (pH adjustment of the sample to analyte in unionized form) of sample, if required.

LLE provide efficient removal of analyte with desired specificity/selectivity required for intended bioanalysis.

Selection of extraction solvent depends on

- Analyte characteristics
- Structure
- pKa
- solubility
- Solvent characteristics as miscibility, purity, efficiency, polarity, inertness, volatility, viscosity, density.

Commonly used extraction solvent are tertiary-Butyl Methyl Ether, Dichloromethane, Ethyl acetate, diethyl ether, hexane etc. any solvent can be used as an extraction solvent.

ADVANTAGE

-Known and standard technique

-Relatively simple to do as a routine

-Trend to micro LLE

-Low LOD's are possible

LIMITATIONS:

-Moderate selective (limited in solvent selections)

-Examine and use pH/ionic strength/temperature

-to get a selective extraction process

-Multiple extractions needed to get the recovery

-Often evaporation steps needed

-Emulsions formed cause recovery loss

-Difficult to automate, semi-automated steps

Protein precipitation

It is basically denaturing the protein, PPT of proteins can be done by using any one of the method mentioned below:

- **By changing the pH of sample-** by adding inorganic reagents eg. Perchloric acid, Trichloroacetic acid etc. At Iso-Electri pH, proteins have no net charge, leads to insolubility thus precipitates.
- **By addition of organic solvents-** it decreases the di-electric constant of the medium, lead to insolubility thus cause precipitation, or high affinity for the hydrophobic surfaces of the protein leads to denaturing of proteins. eg.Methanol, Acetonitrile etc.
- **Salt induced precipitation-** salts used for precipitation of proteins are citrates, phosphates, acetates etc. At high concentration of salts solubility of proteins drop sharply, thus precipitates.

Hybrid Extraction Technique

"Selective extraction of analyte by using the combination of two or more extraction techniques" eg. PPT and SPE or PPT and LLE.

Hybrid extraction is intended for

- Improve specificity
- Improve detection limit
- Improve recovery[22,24,25]

1.6 ANALYTICAL MEHOD VALIDATION[26]

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[18]

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[19].

Data Elements Required for Assay Validation

Both the USP and ICH recognize that is it 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 Characteristics	**Identification**	**Testing for impurities**	**Quantitative Limit**	**Assay -dissolution (measurement only) -content/potency**
Accuracy	-	+	-	+
Precision				
Repeatability	-	+	-	+
Interm.Precision	-	+(1)	-	+(1)
Reproducibility	-	- (2)	-	- (2)
Specificity (3)	+	+	+	+(4)
Detection Limit	-	-	+	-
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 y-intercepts 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 analyzed 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[20].

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 $r^2 > 0.999$
Range	80 –120 %
Stability	> 24 h or >12 h

BIOANALYTICAL METHOD VALIDATION[27,28,29]

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 the sample. 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. A calibration curve should consist of a blank sample (matrix sample processed without internal standard), and six to eight non-zero samples covering the expected range.

The simplest model that adequately describes the concentration-response relationship should be used. Selection of weighting and use of a complex regression equation should be justified. The following conditions should be met in developing a calibration curve:

- 15% deviation of standards other than LLOQ from nominal concentration.

At least four out of six non-zero standards should meet the above criteria, including the LLOQ and the calibration standard at the highest concentration. Excluding the standards should not change the model used.

Accuracy

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 accuracy and precision with which known concentrations of analyte in biological matrix can be determined should be demonstrated. This can be accomplished by analysis of replicate sets of analyte samples of known concentrations (QC samples) from an equivalent biological matrix. At a minimum, three concentrations representing the entire range of the standard curve should be studied: one within 3 X the lower limit of quantification (LLOQ) (low QC sample), one near the center (middle QC), and one near the upper boundary of the standard curve (high QC).

Precision

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. 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.

Selectivity

It 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. It may be important to consider the variability of the matrix due to the physiological nature of the sample. Appropriate steps should be taken to ensure the lack of matrix effects throughout the application of the method, especially if the nature of the matrix changes from the matrix used during method validation.

Recovery

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 un-extracted standards that represent 100% recovery.

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. 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.

A. Short-Term Temperature Stability

Three aliquots of each of the low and high concentrations should be thawed at room tempserature and kept at this temperature from 4 to 24 hours (based on the expected duration that samples will be maintained at room temperature in the intended study) and analyzed.

B. Freeze and Thaw Stability

Analyte stability should be determined after three freeze and thaw cycles. At least three aliquots at each of the low and high concentrations should be stored at the intended storage temperature for 24 hours and thawed unassisted at room temperature. When completely thawed, the samples should be refrozen for 12 to 24 hours under the same conditions. The freeze-thaw cycle should be repeated two more times, and then analyzed on the third cycle.

C. Long-Term Stability

The storage time in a long-term stability evaluation should exceed the time between the date of first sample collection and the date of last sample analysis. Long-term stability should be determined by storing at least three aliquots of each of the low and high concentrations under the same conditions as the study samples. The volume of samples should be sufficient for analysis on three separate occasions. The concentrations of all the stability samples should be compared to the mean of back-calculated values for the standards at the appropriate concentrations from the first day of long-term stability testing.

D. Stock Solution Stability

The stability of stock solutions of drug and the internal standard should be evaluated at room temperature for at least 6 hours. If the stock solutions are refrigerated or frozen for the relevant period, the stability should be documented. After completion of the desired storage time, the stability should be tested by comparing the instrument response with that of freshly prepared solutions.

Chapter 2
Literature review

Table 2.1 Determination of MEM

S. No.	Matrix	Method	Condition	Validation parameters	Ref. No.
1.	Tablet	UV-Visible Spectroph-otometric	Ion-pair complexes of the drug with Bromocresol Green (BCG: λmax 415 nm) and Bromothymol Blue (BTB : λmax 420 nm) Solvent : distilled water	Linearity - 4-12 µg/ml (R^2 = 0.9990) and 2-6 µg/ml (R^2 0.9995) respectively for BCG and BTB	30
2.	Tablet	UV-Visible Spectroph-otometric	Complex formation with iodine after conversion in free base by reaction with ammonia (λmax 291 nm) Solvent : distilled water	Linearity - 10-30 µg/ml (R^2 = 0.9999)	31
3.	Tablet	UV-Visible Spectroph-otometric	A. Reduction of F. C. ragent by drug (λmax760 nm) B. Condensation of drug with NQS reagent (λmax 460 nm) Solvent : distilled water	Linearity – A. 4-12 µg/ml (R^2 = 0.997) B. 7.5 – 17.5 µg/ml (R^2 = 0.999)	32
4.	Tablet	UV-Visible Spectroph-otometric	Ion-associate complexes of drug with BCG (λmax 415 nm) , BPB (λmax 412 nm) and BTB (λmax 414 nm) Solvent : double distilled water	Linearity – 1.5–16.5 µg/ml (R^2 = 0.9998), 1.4–14 µg/ml (R^2 = 0.9996) 1.6–17 µg/ml (R^2 = 0.9997) respectively for BCG, BPB, BTB	33

5.	Tablet	UV-Visible Spectroph-otometric and spectroflu-orimetric	A. Reaction of the drug with 4 -chloro-7-nitro-2,1,3-benzo xadiazole in alkaline buffer (λmax 476 nm, λexci 455 nm and λemi 500 nm) Solvent : methanol and acetone B. Reaction of drug with o-phthalaldehyde/ N-acetyl-L-cysteine (λmax 340 nm) Solvent: distilled water	Linearity – A. 5-70 µg/ml (R^2 = 0.9999) and 0.02 – 0.2 µg/ml (R^2 = 0.9999) B. 5-50 µg/ml (R^2 = 0.9994)	34
6.	Capsul e	UV-Visible Spectroph-otometric and spectroflu-orimetric	Condensation of drug with NQS in an alkaline medium A. (λmax 460 nm) B. (λexci 293nm, λemi 382 nm) Solvent : distilled water	Linearity – A. 5 -80 µg/ml (R^2 = 0.9972) B. 5 -80 µg/ml (R^2 = 0.9974)	35
7.	Tablet	Spectroflu-orimetric	Oxidation of drug by cerium(IV) in presence of perchloric acid and subsequent moni-toring the fluorescence of the induced cerium(III) (λexci 255 nm and λemi 355 nm) solvent : 0.1N sulphuric acid	Linearity – 50–1200 ng/ml (R^2 = 0.9996)	36

8.	Plasma	RP-HPLC	C_{18} column mobile phase consisting of 80% acetonitrile and 20% phosphate buffer solution pre-column derivatization with 0.015M 9-fluorenylmethyl chloroformate and 0.5 M borate buffer solution (λmax 265 nm) Flow rate : 0.8 mL/ minute	Linearity – 70-130µg/ml (R^2 = 0.999)	37
9.	Plasma	RP-HPLC	C_{18} column mobile phase of phosphate buffer and acetonitrile pre-column derivatization with Dansyl chloride (λmax 265 nm) Flow rate : 1.5 mL/minute	Linearity – 3–400 ng / ml (R^2 = 0.999)	38
10.	Plasma	RP-HPLC	reversed-phase C_{18} column mobile phase of phosphate buffer and acetonitrile pre-column derivatization with OPA (λexci 335 nm and λemi	Linearity – 2–80 ng / ml (R^2 = 0.99986)	39

			440 nm)		
11.	Plasma	LC-MS/MS	C_{18} column mobile phase composed of methanol–water–formic acid (80:20:0.1, v/v/v)	Linearity – 0.1–50.0 ng / ml ($R^2 \geq 0.98$)	40

Table 2.2 Determination of DH

S. No.	Matrix	Method	Condition	Validation parameters	Ref. No.
1.	Tablet	UV-Visible Spectroph-otometric	Based on the reduction of potassium permanganate in alkaline medium with drug (λmax 547nm) Solvent : distilled water	Linearity – 2-35 µg/ml (R^2 = -0.98983)	41
2.	Tablet	UV-Visible Spectroph-otometric	Ion pair complex of drug with Eriochrome Black T dye (λmax 510nm) Solvent : Deionized water	Linearity – 0.2-25µg/ml (R^2 = 0.995)	42
3.	Tablet	UV-Visible Spectropho t-ometric and spectrofluo	A. λmax 315 nm B. λexci 226 nm and λemi 391 nm	A. Linearity 8—56 µg/ml (R^2 = 0.9998) B. Linearity –	43

		ri-metric	Solvent : Deionized water	0.32— 3.2µg/ml (R^2 = 0.9993)	
4.	Tablet	RP-HPLC	C_{18} column Mobile phase - methanol, phosphate buffer 0.02 M and triethylamine (50:50:0.5) Detection λ 268 nm Flow tare 1 mL/minute	Linearity 10 to 60µg/ml (R^2 = 0.9995)	44
5.	Tablet	RP-HPLC	C_8 column Mobile phase - buffer : methanol : triethylamine (550:450:5) Detection λ 271 nm Flow rate 1 mL/minute	Linearity 20 to 60µg/ml (R^2 = 0.9996)	45
6.	Tablet	RP-HPLC	C_{-18} column Mobile phase - 0.005 M K2 HPO4: methanol (38:62) Detection λ 270 nm Flow tare 1 mL/minute	Linearity 0.35– 0.64mg/ml (R^2 = 0.9980)	46
7.	Tablet	RP-HPLC	Phenyl RP column Mobile phase - methanol, 0.02 M phosphate Buffer (pH 7.5 ± 0.1) and triethylamine in the ratio 60: 40: 0.5 Detection λ 268 nm Flow tare 1 mL/minute	Linearity 50 -150 µg/ml (R^2 = 0.997)	47

8.	Tablet	RP-HPLC	C_{18} column Mobile phase - methanol, water (60:40) Detection λ 230 nm Flow tare 1 mL/minute	Linearity 2 – 60 µg/ml (R^2 = 0.998)	
9.	Plasma and Tablet	RP-HPLC	C_{18} column methanol, phosphate buffer (0.02 mol L–1) and triethyl amine (pH 3.5) (55 : 45 : 0.5) Detection λexci 290 nm & λemi 315nm Flow tare 0.9 mL/minute	Linearity 5–2000 ng/ml (R^2 =0.998)	48
10.	Tablet	HPTLC	silica gel 60 F 254 on aluminium sheet mobie phase - Methanol: Chloroform (8:2 v/v) Detection λ 254 nm	Linearity 200-1000 ng/spotl (R^2 =0.9995)	49
11.	Plasma	LC-MS/MS	C_{-18} column Mobile phase - 75%10 mM formic acid and 25% methanol Flow rate : 1 mL/minute	Linearity - 0.0206 - 51.6 ng / ml ($R^2 \geq 0.997$)	50
12	Plasma	RP-HPLC	cellulose tris (3,5-dimethylphenyl carbamate) column Mobile phase - n-hexane, isopropanol and triethylamine (87:12.9:0.1) detection λ 268 nm	Linearity 0.05–2 µg/ml ($R^2 \geq 0.994$)	51

			Flow rate : 1.5 mL/minute		
13	Plasma	RP-HPLC	C_{-18} column Mobile phase - phosphate buffer (0.02 M, pH 4.6), perchloric acid (6 M) and acetonitrile (59.5:0.5:40, v / v) detection λ 315 nm Flow rate : 1 mL/minute	Linearity 3–90ng/ml (R^2 = 0.9987)	52
14.	Plasma	RP-HPLC	C_{30} column Mobile phase - 25 mM citric acid/50 mM Na_2 HPO_4 (pH 6.0)–CH_3 CN (73:27%, v/v) detection λexci 325 nm, λemi 390 nm Flow rate : 1.5 mL/minute	Linearity 1-50ng/ml (R^2 = 0.999)	53
15.	Plasma	LC-MS	C_{18} column Mobile phase - 0.2% formic acid in 20 mM ammonium acetate–methanol–acetonitrile (63:20:17, v/v/v	Linearity 0.10–50.0 ng/ml (R^2 = 0.9975)	54
16.	Plasma	LC-MS	C_{18} column Mobile phase- 82% acetonitrile and 18% 10 mM ammonium acetate Flow rate : 1 mL/minute	Linearity 0.1–100 ng/ml (R^2 = 0.999)	55

Table 2.3 Determination of MEM & DH

S. No.	Matrix	Method	Condition	Validation parameters	Ref. No.
1.	Plasma	UPLC-MS/MS	C_{18} column Mobile phase- gradient elution of an ammonium acetate buffer at pH 9.3 and acetonitrile Flow rate : 1.5 mL/minute	A. MEM Linearity 1–300 ng/ml (R^2 = 0.9998) B. DH Linearity 2–300 ng/ml (R^2 = 0.9997)	56
2.	Mixture	HPTLC	Silica gel 60 HPTLC plate Mobile phase – chloroform : cyclohexane : methanol (2:2:1) detection λ 274 nm	A. MEM Linearity 5-30µg B. DH Linearity 1-20 µg	57

Chapter 3
Aim and Objective of Work

Aim of the Present Work

Alzheimer's disease is a progressive neurologic disease of the brain leading to the irreversible loss of neurons. Few drugs are available to slow down the progress of disease, mainly cholinesterase inhibitors (Donepezil Hydrochloride) and NMDA anatagonists (Memantine Hydrochloride).

DH and MEM both drugs are used as combination therapy recently for treatment of AD. Literature review revealed that only one HPTLC and one LC-MS/MS method has been reported for simultaneous estimation of DH and MEM in synthetic mixture and in plasma respectively.

However, there is no method reported till now for the simultaneous estimation of these drugs in a combined dosage formulation. Hence it was endeavored to develop validated analytical methods for simultaneous estimation of these drugs in combined dosage formulation and to develop a simple, cost effective method for estimation of these drugs in human plasma.

Objectives of the Present Work

The objectives of the work envisaged are:

- To develop UV spectrophotometric, spectrofluorimetric methods for simultaneous estimation of DH and MEM in combine tablet dosage form.
- Validate the developed methods as per ICH guidelines.
- To develop and validate spectrofluorimetric method for estimation of DH and MEM in human plasma.

Chapter 4
Experimental work

4.1 IDENTIFICATION

INSTRUMENTATION

- Melting Point Apparatus- T603160, (EIE Instruments, Pvt. Ltd.)
- UV/Vis-2400, Version-2.21 double beam spectrophotometer with spectral width of 2 nm, wavelength accuracy of 0.5 nm and a pair of 10 mm matched quartz cells (Shimadzu, Columbia, MD)
- JASCO FT/IR-6100, (Inc. Japan) TGS Detector with Spectra Manager Software

MELTING POINT DETERMINATION

Melting Point of pure drugs has been determined using capillary melting point apparatus. Melting Points obtained were compared with that available in literature as shown in table11**.**

Table 4.1: Melting point of pure drugs

DRUG	REPORTED[18]	OBSERVED
MEM	290 - 295 ^{0}c	293 - 295^{0}c
DH	211- 212^{0}c	212 - 215^{0}c

DETERMINATION OF UV SPECTRA

The UV spectra of DH (10 µg/mL) solution was taken in the range of 200-400 nm as shown in figure 6 and. From these spectra, λ_{max} of DH drug was obtained and compared with that available in Literature as shown in table 12.

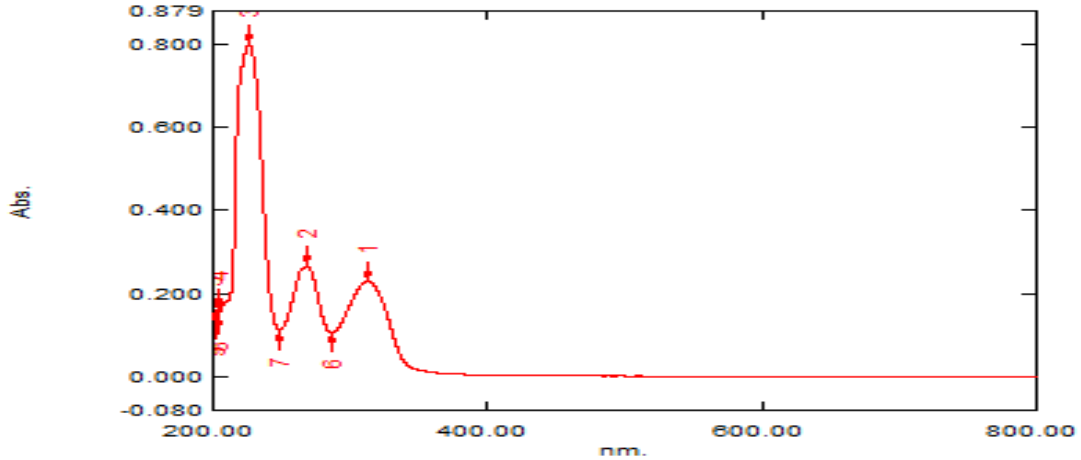

Fig.4.1: UV-spectra of DH (10 µg/mL) in methanol

Table 4.2: Comparison of observed peaks of DH with reported peaks

Reported peaks[11]	Observed peaks
230 nm	230 nm
268 nm	267.8 nm
313 nm	312.6 nm

4.1.4 Determination of FT-IR Spectra- IR Spectra of MEM and DH were taken by FT-IR.

4.1.4.1 Determination of FT-IR Spectra of DH- IR Spectra of DH is shown in figure 4.3. The specification of Peaks is given in table 4.3.

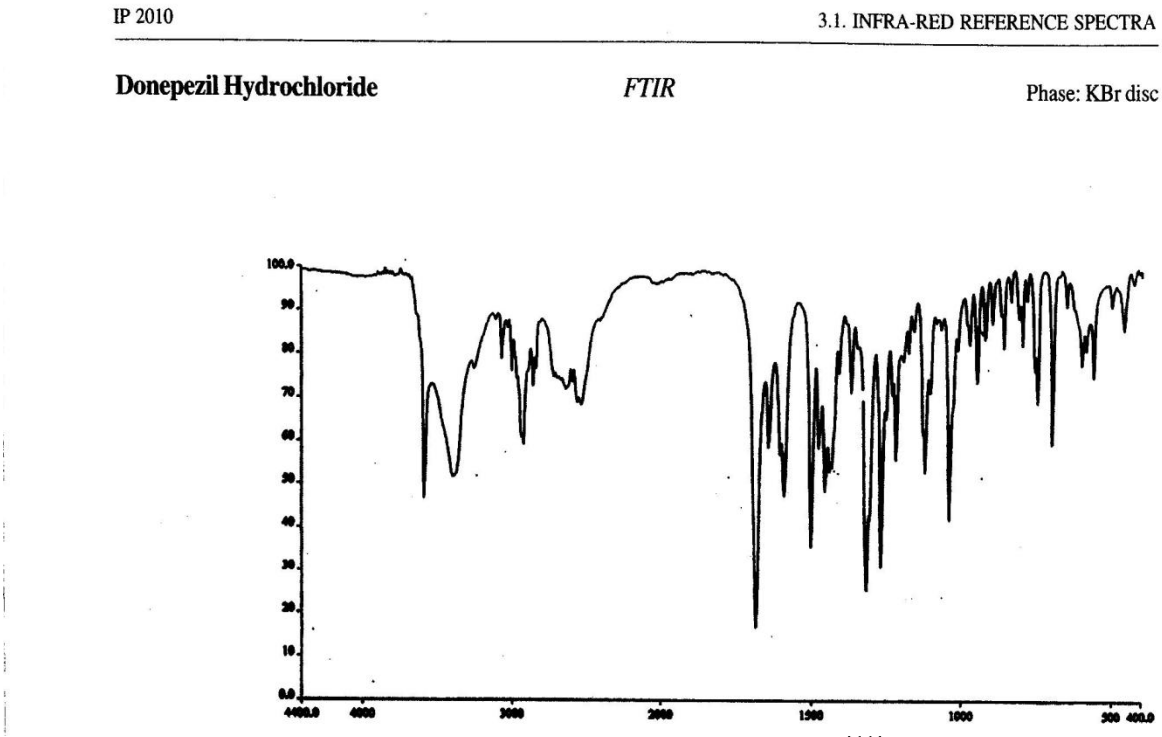

Fig.4.2: Reported FT-IR spectra of DH[11]

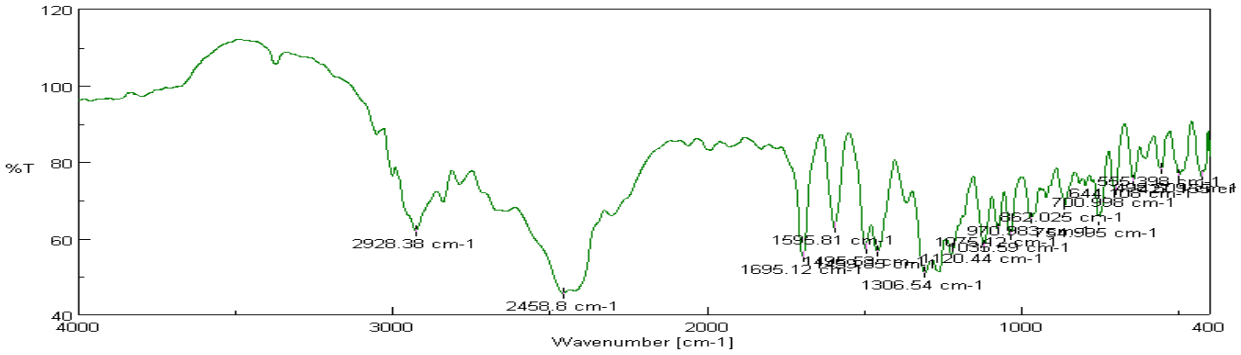

Fig 4.3 : Observed FT-IR spectra of DH

Table 4.3: specification of FT-IR spectra of DH

WAVE NO. (cm^{-1})	FUNCTIONAL GROUP
1695	Carbonyl group
2928	Aromatic hydrogen stretching
1495	C=C stretching
1454	$-CH_2$ deformation

4.1.4.2 Determination of FT-IR Spectra of MEM - IR Spectra of MEM is shown in figure 12. The specification of Peaks is shown in table16

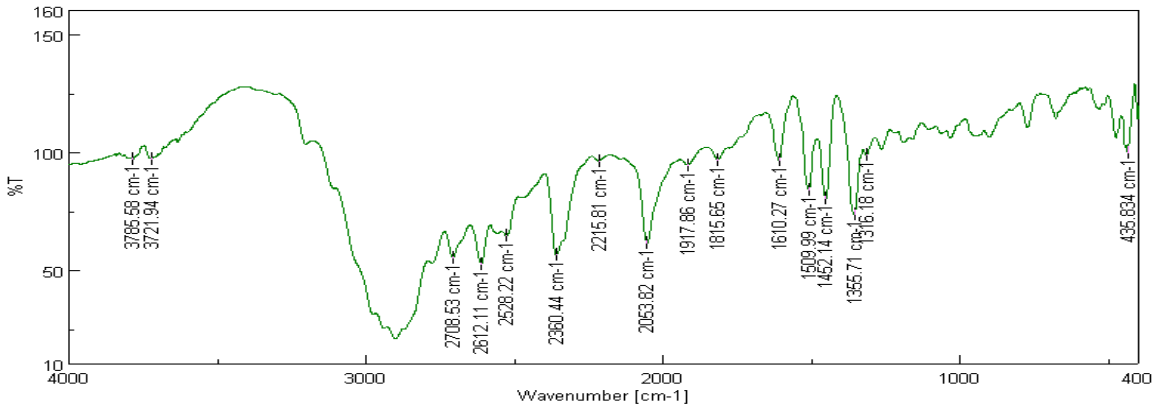

Fig 4.4: Observed FT-IR-spectra of MEM

Table 4.4: specification of FT-IR spectra of MEM

WAVE NO. (cm^{-1})	**FUNCTIONAL GROUP**
3300	Primary amine stretching
1452	Methyl –CH stretching
2901	Cyclohexane

4.2 DEVELOPMENT AND VALIDATION OF UV SPECTROPHOTOMETRIC METHOD FOR SIMULTANEOUS ESTIMATION OF MEMANTINE HYDROCHLORIDE AND DONEPEZIL HYDROCHLORIDE

4.2.1 INSTRUMENT

- **UV-Visible Double-Beam spectrophotometer** Model: UV-2450 PC series, Matched quartz cell (1cm), Manufacturer: Shimadzu Inc. Japan, Wavelength range: 200.00 to 800.00 nm
- **Analytical Balance** Model: CX -220, Citizen
- **Sonicator** Model: TRANS-O-SONIC; D-compect., Capacity: 2 Lit.
- **pH meter** Model: digital pH meter, Analab scientific Instrumentation Pvt. Ltd.
- **Water bath** Model: Water bath with digital temperature controller, EIE Instrument Pvt. Ltd.
- **Hot air oven** Model: Hot air oven with digital temperature controller, EIE Instrument Pvt. Ltd.

4.2.2 REAGENT AND MATERIAL

- API Memantine Hydrochloride (MEM) gifted sample from INTAS Pharmaceuticals Pvt. Ltd.
- API Donepezil Hydrochloride (DH) gifted sample from INTAS Pharmaceuticals Pvt. Ltd.
- Methanol (AR Grade, S.D.Fine Chemicals Ltd., Bombay, India)
- Ortho-pthaladehyde (AR Grade, Sisco Research Laboratories Pvt ltd.)
- β – mercaptoethanol (for molecular biology, AR Grade, Sisco Research Laboratories Pvt ltd.)
- Boric acid (AR Grade, S.D.Fine Chemicals Ltd., Bombay,India)
- Potassium chloride (AR Grade, S.D.Fine Chemicals Ltd., Bombay,India)
- Sodium hydroxide (AR Grade, S.D.Fine Chemicals Ltd., Bombay, India)
- Tablets: Containing Memantine Hydrochloride (5mg) and Donepezil Hydrochloride (5mg) Brand name : ALZIL-M5 (INTAS) purchased from Ahmedabad.

4.2.3 METHOD

4.2.3.1 PREAPARATION OF SOLUTIONS

4.2.3.1.1 Preparation of standard stock solution of MEM and DH

MEM (25 mg) and DH (25mg) were accurately weighed and transferred to two separate 25 mL volumetric flasks. Dissolved in 10 mL methanol, sonicated for 10 min and volume made up to mark with methanol to obtain standard stock solution having concentration 1000 µg/mL each. Each the aliquot 5ml from this solution was transferred 50 mL volumetric flask and volume made up with methanol to obtain working standard stock solution of 100 µg/mL.

4.2.3.1.2 Preparation of sample Solution

Quantity of tablet powder (205.37 mg) equivalent to 5 mg of MEM (and 5 mg of DH) was weighed and transferred to a 25 mL volumetric flask containing about 15 mL of methanol, ultrasonicated for 5 min, filtered, filter was washed with methanol, combined with the filtrate and volume was made up to the mark with the methanol to get sample stock solution of 200 µg/mL of MEM and DH.

4.2.3.1.3 Preparation of Borate buffer pH (9.6)

Boric acid (3.09 gm) and Potassium Chloride (3.73 gm) were accurately weighed and transferred to 250 mL volumetric flask. Volume was made up with distilled water. Then solution was transferred to 1000 mL beaker and pH of solution adjusted to 9.6 by addition of 0.2 M of Sodium Hydroxide solution. After pH adjustment volume was made up 1000mL with distilled water.

4.2.3.1.4 Preparation of derivatization reagent

OPA (50 mg) was taken in 25 mL amber colored volumetric flask, 5 mL methanol was added, sonicated for 5 minutes. β-mercaptoethanol (20 µL) was added and finally volume was made up to mark with borate buffer pH 9.6 and store at temperature below 10^0c.

4.2.3.2 DERIVATIZATION METHOD

Each solution of both standard as well as sample of both the drugs was prepared by derivatization method. In derivatization method, aliquot of drug soution was taken in 10mL volumetric flask. 0.1 mL of deivatization reagent solution was added. The solution was kept on water bath for 10 minutes at 70^0c. Then volume was made up with methanol and solutions were

kept at room temperature for next 10 minutes. The solution was scanned and this can be done within 2 hrs at room temperature or within 12 hrs if solutions stored below 10^0c.

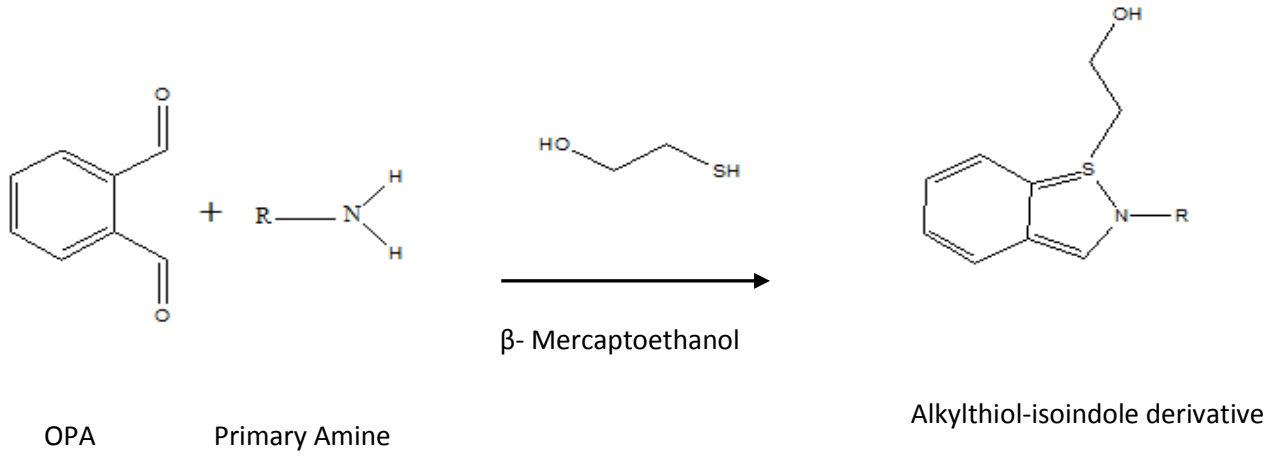

Fig. 4.5 : Reaction pathway of derivatization of primary amine with OPA

4.2.3.3 OPTIMIZATION OF EXPERIMENTAL CONDITIONS

- Concentration of OPA : 0.2 % w/v
- Concentration of β- mercaptorthanol : 0.08 % V/V
- pH of borate buffer : 9.6
- Dilution Solvent : Methanol
- Reaction time : 10 minutes
- Reaction temperature : 70^0C

4.2.3.4 SELECTION OF ANALYTICAL WAVELENGTH

From the working standard solutions, 30 µg/mL solutions were prepared by transferring 3 mL from working standard solution of both the drugs in two different 10 mL volumetric flasks respectively, deivatization reagent solution (0.1 mL) was added in volumetric flasks and was kept in water bath for 10 minutes at 70^0C. Then volume was made up with methanol and solution were kept at room temperature for 10 minutes. Solutions were scanned in the spectrum mode from 200 to 400 nm. From the overlain spectra of these drugs, wavelengths 312.60 nm (λ_{max} of DH) and 337 nm (λ_{max} of MEM) were selected for simultaneous analysis of both the drug using simultaneous equation method.

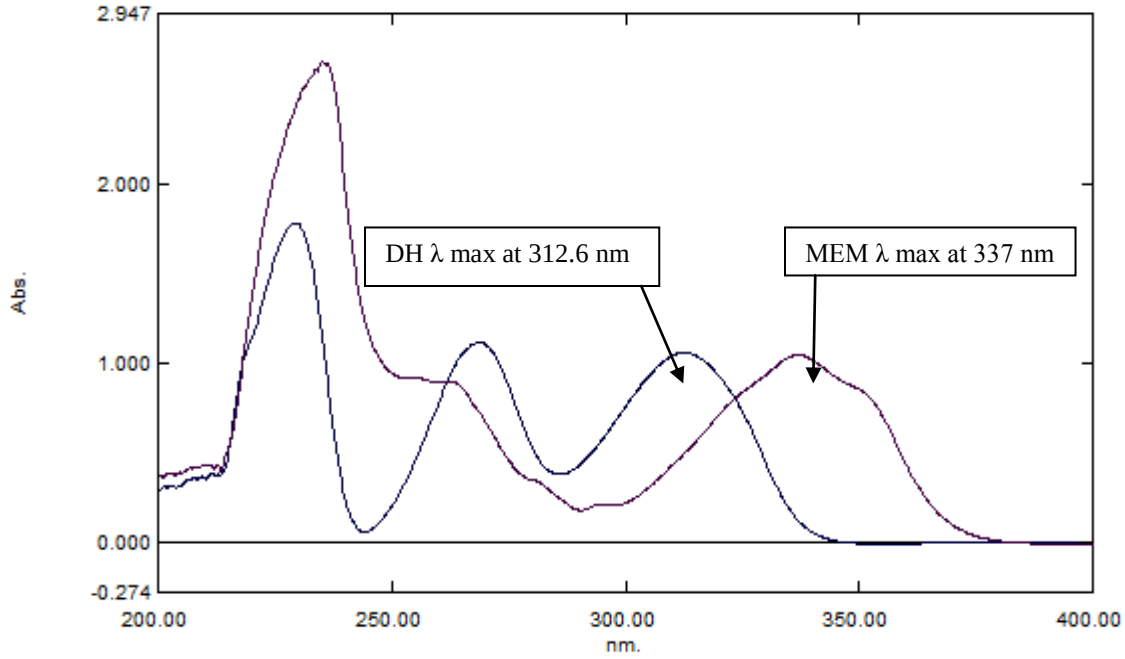

Fig. 4.6: Overlay spectra of MEM and DH (30µg/mL) in methanol

4.2.4 METHOD VALIDATION

4.2.4.1 Linearity

Preparation of calibration curve

For calibration curve of both the drugs 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 mL were taken from standard stock solution in 10 mL volumetric flasks respectively. Derivatization reagent solution (0.1 mL) was added in volumetric flask and further same procedure was followed as per section 4.2.3.2**.** After that solutions were scanned for absorbance on 312.6 nm and 337 nm and scanning can be done within 2 hrs at room temperature or within 12 hrs if solutions stored below 10^0c.

4.2.4.2 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).

4.2.4.2.1 Intra-day and Interday precision

Intra-day and Inter-day precision was determined by measuring the absorbance of both the drugs three times within a day and on three different days, respectively. For intraday and interday precision 1.0, 4.0, 7.0 mL were taken from working standard stock solution in 10 mL

volumetric flasks to obtain conc. of 10, 40, 70 µg/ mL for both the drug respectively and 0.1 mL of derivatization reagent solution was added in volumetric flask and further same procedure was followed as per section 4.2.3.2**.** After that solution were scanned for absorbance on 312.6 nm and 337 nm.

4.2.4.2.2 Repeatability

It is a measure of precision under the same operating conditions over a short interval of time. It is sometimes referred to as intra-assay precision. To study the repeatability, six determinations at 100% test concentrations (i.e. 30µg/mL for both MEM and DH) were carried out. For repeatability study 3.0 mL was taken from working standard stock solution in 10 mL volumetric flasks and 0.1 mL of derivatization reagent solution was added in volumetric flask and further same procedure was followed as per section 4.2.3.2**.** After that solution were scanned for absorbance on 312.6 nm and 337 nm.

4.2.4.3. Limit of detection and limit of quantification (LOD/LOQ)

For this determination Calibration curve for both the drugs was repeated six times 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 the Intercept

S = slope of calibration curve

4.2.4.4. Robustness-

Robustness was performed on concentrations (30 µg/mL) of MEM and (30 µg/mL) of DH. The solutions were prepared as per section 4.2.3.2. Robustness of the method was determined by making change in λ_{max} of both the drugs by ±2 nm. The % Assay values were calculated and compared with that of standard. Results were reported in terms of % RSD.

4.2.4.5. Accuracy

Accuracy was determined in terms of % recovery. Recovery study was done by standard addition method. To study the accuracy, 10 tablets were weighed and powdered. Quantity of tablet powder (205.37 mg) equivalent to 5 mg of MEM (and 5 mg of DH) was weighed and transferred to a 25 mL volumetric flask containing about 15 mL of methanol, ultrasonicated for 5 min, filtered, filter was washed with methanol, combined with the filtrate and volume was made up to the mark with the methanol to get sample stock solution of 200 µg/mL of MEM and DH.

For MEM accuracy studies, 1.5 mL was taken from 200 µg/mL tablet sample solution in three different 10 mL volumetric flasks and 2.4 mL, 3.0 mL, 3.6 mL (80%, 100% and 120% of test sample concentration) of working standard MEM solution was added and followed by 0.1 mL of derivatization reagent solution was added in volumetric flask and further same procedure was followed as per section 4.2.3.2**.** After that solution were measured for absorbance at 312.6 nm and 337 nm.

For DH accuracy studies, 1.5 mL was taken from 200 µg/mL sample solution in three different 10 mL volumetric flask and 2.4 mL, 3.0 mL, 3.6 mL (80%, 100% and 120% of test sample concentration) was taken from working standard solution in 10 mL volumetric flask and 0.1 mL of derivatization reagent solution was added in volumetric flask and further same procedure was followed as per section 4.2.3.2. After that solution were scanned for absorbance on 312.6 nm and 337 nm.

4.2.4.6 ANALYSIS OF MEM AND DH IN COMBINATION TABLET DOSAGE FORM

Form the sample solution (prepared as per section 4.2.3.1.2), 1.5 mL solution was taken in 10 mL volumetric flask and further same procedure was followed as per section 4.2.3.2**.** After that solution were scanned for absorbance on 312.6 nm and 337 nm.

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4.3 DEVELOPMENT AND VALIDATION OF SPECTROFLUORIMETRIC METHOD FOR SIMULTANEOUS ESTIMATION OF MEMANTINE HYDROCHLORIDE AND DONEPEZIL HYDROCHLORIDE

4.3.1 INSTRUMENT

- **Spectrofluorimeter** Model: FP- 6500 PC series, Matched quartz cell (1cm), Manufacturer JASCO Japan, Wavelength range: 220.00 to 750.00 nm
- **Analytical Balance** Model: CX -220, Citizen
- **Sonicator** Model: TRANS-O-SONIC; D-compect, Capacity: 2 Lit.
- **pH meter** Model: digital pH meter, Analab scientific instrumentation Pvt. Ltd.
- **Water bath** Model: Water bath with digital temp. controller, EIE instrument Pvt. Ltd.
- **Hot air oven** Model: Hot air oven with digital temp. controller, EIE instrument Pvt. Ltd.

4.3.2 MATERIALS

- API Memantine Hydrochloride (MEM) gifted sample from INTAS pharmaceuticals Pvt. Ltd.
- API Donepezil Hydrochloride (DH) gifted sample from INTAS pharmaceuticals Pvt. Ltd.
- Methanol (AR Grade, S.D.Fine Chemicals Ltd., Bombay, India)
- OPA (AR Grade, Sisco Research Laboratories Pvt Ltd.)
- β – mercaptoethanol (for molecular biology, AR Grade, Sisco Research Laboratories Pvt. Ltd.)
- Boric acid (AR Grade, S.D.Fine Chemicals Ltd., Bombay, India)
- Potassium chloride (AR Grade, S.D.Fine Chemicals Ltd., Bombay, India)
- Sodium hydroxide (AR Grade, S.D.Fine Chemicals Ltd., Bombay, india)
- Tablets: containing Memantine Hydrochloride (5mg) and Donepezil Hydrochloride (5mg) Brand name : ALZIL-M5 (INTAS) purchased from Ahmedabad.

4.3.3 METHOD

4.3.3.1 PREAPARATION OF SOLUTIONS

4.3.3.1.1 Preparation of standard stock solution of MEM and DH

MEM (25 mg) and DH (25mg) were accurately weighed and transferred to two separate 25 mL volumetric flasks. Dissolved in 10 mL double distilled water, sonicated for 10 min and volume made up to mark with double distilled water to obtain standard stock solution having concentration 1000 µg/mL each. 5 mL aliquot from these solutions were transferred to two 50 mL volumetric flasks, respectively and volume was made up to 25 mL with double distilled water to obtain working standard stock solution of 100 µg/mL.

4.3.3.1.2 Preparation of sample Solution

Quantity of tablet powder (205.37 mg) equivalent to 5 mg of MEM (and 5 mg of DH) was weighed and transferred to a 25 mL volumetric flask containing about 15 mL of double distilled water, ultrasonicated for 5 min, filtered, filter was washed with double distilled water, combined with the filtrate and volume was made up to the mark with the double distilled water to get sample stock solution of 200 µg/mLof MEM and DH.

4.3.3.1.3 Preparation of Borate buffer pH (9.6)

Boric acid (3.09 gm) and Potassium Chloride (3.73 gm) were accurately weighed and transferred to 250 mL volumetric flask. Volume was made up with double distilled water. Then solution was transferred to 1000 mL beaker and pH of solution adjusted to 9.6 by addition of 0.2 M of Sodium Hydroxide solution. After pH adjustment volume was made up to 1000mL with double distilled water.

4.3.3.1.4 Preparation of derivatization reagent

OPA (50 mg) was taken in 25 mL amber colored volumetric flask, 5 mL methanol was added, sonicated for 5 min. β-mercaptoethanol (20 µl) was added and finally volume was made up to mark with borate buffer pH 9.6 and store at temperature below 10^0c.

4.3.3.2 DERIVATIZATION OF SAMPLES

Each solution of both standard as well as sample of both the drugs was prepared by same derivatization method. In derivatization method aliquot of drug soultion was taken in 10mL volumetric flask and then derivatization reagent solution (0.1 mL) was added. The solution was kept on water bath for 10 min at 70^0C. Then volume was made up with borate buffer and

solution was kept at room temperature for 10 minutes. The solution was scanned within 10 minutes.

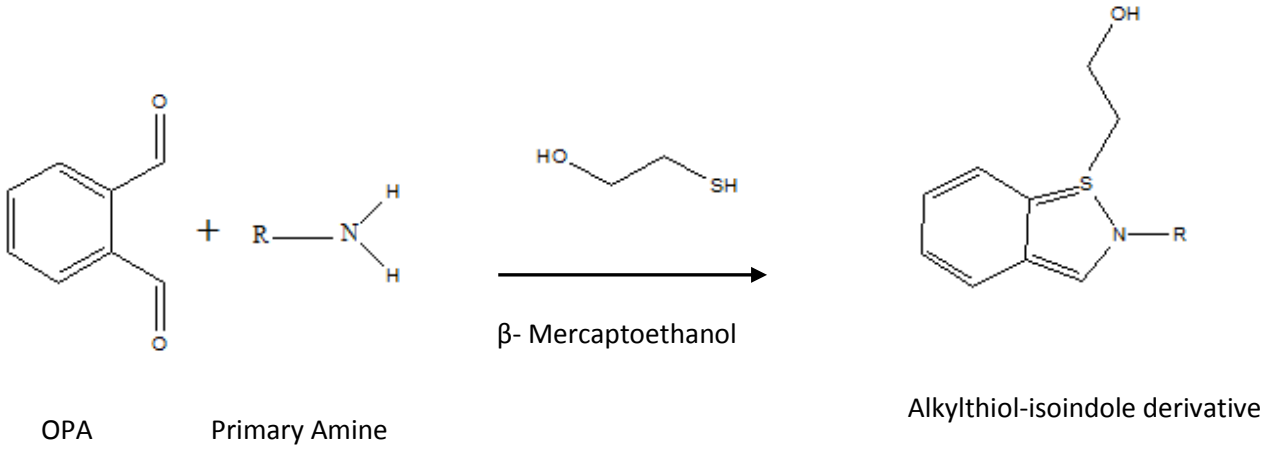

Fig. 4.17: Chemical reaction of derivatization of primary amine

4.3.3.3 OPTIMIZATION OF EXPERIMENTAL CONDITIONS

- Excitation and emission wavelength
- Delta value : (65nm)
- Dilution solvent : Borate buffer (pH 9.6)
- Sensitivity : Medium

4.3.3.4 SELECTION OF ANALYTICAL WAVELENGTH

From the working standard solutions, 10 µg/mL solutions of both the drugs were prepared by transferring 1 mL in 10 mL volumetric flask and derivatization reagent solution (0.1 mL) was added in volumetric flask and further same procedure was followed as per section 4.3.3.2. After that solution were scanned in the synchronous mode with delta value 66 (low sensitivity mode) from 220 to 680 nm. From the overlain synchronous spectra of these drugs wavelengths 361 nm (zero crossing point for MEM) selected for estimation of DH and 450 nm (zero crossing point for DH) for MEM were selected for analysis.

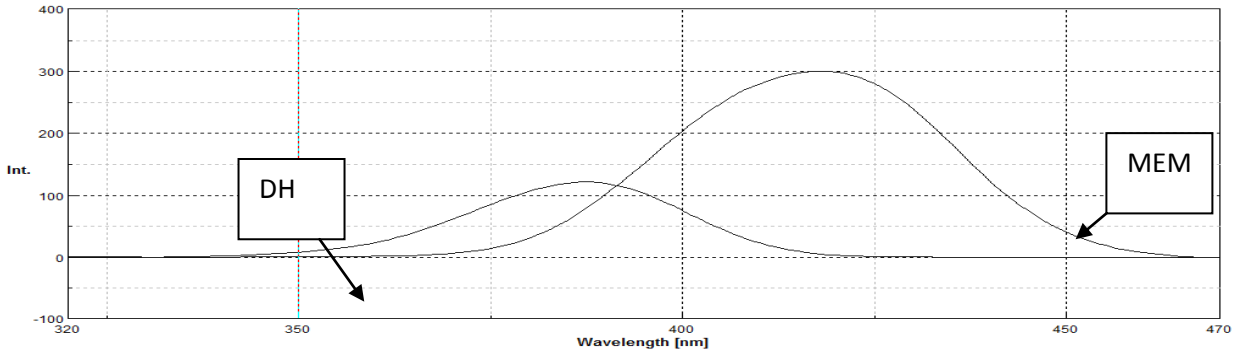

Fig. 4.18: Overlay synchronous spctrofluorimetric spectra of MEM and DH in borate buffer

4.3.4 METHOD VALIDATION

4.3.4.1 Linearity

Preparation of calibration curve

For calibration curve of both the drugs 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mL were taken from working standard stock solution in 10 mL volumetric flasks and 0.1 mL of derivatization reagent solution was added in volumetric flask and further same procedure was followed as per section 4.3.3.2. After that solution were scanned.

4.3.4.2 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).

4.3.4..2.1 Intra-day precision and Inter-day precision

Intra-day and Inter-day precision was determined by measuring the absorbance of both the drugs three times within a day and on three different days, respectively. For intraday and interlay precision 0.5, 1.5, 2.5 mL were taken from working standard stock solution in 10 mL volumetric flasks to obtain conc. of 15, 25 µg/mL for both drugs and 0.1 mL of deivatization reagent solution was added in volumetric flask and further same procedure was followed as per section 4.3.3.2. After that solution were scanned.

4.3.4.2.2 Repeatability

Repeatability is a measure of precision under the same operating conditions over a short interval of time. It is sometimes referred to as intra-assay precision. To study the repeatability, six determinations at 100% test concentrations (i.e.15µg/mL for both MEM and DH) were carried out. For repeatability 1.5 mL was taken from working standard stock solution in 10 mL

volumetric flasks and 0.1 mL of derivatization reagent solution was added in volumetric flask further same procedure was followed as per section 4.3.3.2. After that solution were scanned.

4.3.4.3 Robustness

Robustness was performed on concentrations (15 µg/mL) of MEM and (15 µg/mL) of DH. The solutions were prepared as per section 4.3.3.2. Robustness of the method was determined by making change in m_{ix} of both the drugs by ±2 nm. The % Assay values were calculated and compared with that of standard. Results were reported in terms of % RSD.

4.3.4.4 Limit of detection and limit of quantification (LOD/LOQ)

For this determination Calibration curve for both the drugs was repeated six times 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 the Intercept

S = slope of calibration curve

4.3.4.5 Accuracy

Accuracy was determined in terms of % recovery. Recovery study was done by standard addition method. To study the accuracy, 10 tablets were weighed and powdered. Quantity of tablet powder (205.37 mg) equivalent to 5 mg of MEM (and 5 mg of DH) was weighed and transferred to a 25 mL volumetric flask containing about 15 mL of double distilled water, ultrasonicated for 5 min, filtered, filter was washed with double distilled water, combined with the filtrate and volume was made up to the mark with the methanol to get sample stock solution of 200 µg/mL of MEM and DH.

For MEM accuracy studies, 0.5 mL was taken from 200 µg/mL tablet sample solution in three different 10 mL volumetric flasks and 0.8 mL, 1.0 mL, 1.2 mL (80%, 100% and 120% of test sample concentration) of working standard MEM solution was added and followed by 0.1 mL of derivatization reagent solution was added in volumetric flask and further same procedure was followed as per section 4.3.3.2**.** After that solution were scanned in spectrofluorimeter.

For DH accuracy studies, 0.5 mL was taken from 200 µg/mL sample solution in three different 10 mL volumetric flask and 0.8 mL, 1.0 mL, 1.2 mL (80%, 100% and 120% of test sample concentration) was taken from working standard solution in 10 mL volumetric flask and 0.1 mL of derivatization reagent solution was added in volumetric flask and further same procedure was followed as per section 4.3.3.2**.**. After that solution were scanned in spectrofluorimeter.

4.3.4.6 ANALYSIS OF MEM AND DH IN COMBINATION TABLET DOSAGE FORM

Form the sample solution was prepared as per section 4.3.3.1.2 , 0.5 mL from this solution was taken in 10 mL volumetric flask and further same procedure was followed as per section 4.3.3.2**.** After that solution were scanned in synchronous mode in spectrofluorimeter.

4.4 DEVELOPMENT AND VALIDATION OF SPECTROFLUORIMETRIC METHOD FOR OF MEMANTINE HYDROCHLORIDE AND DONEPEZIL HYDROCHLORIDE IN HUMAN PLASMA

4.4.1 INSTRUMENTS

- **Spectrofluorimeter** Model: FP- 6500 PC series, Matched quartz cell (1cm), Manufacturer JASCO Japan, Wavelength range: 220.00 to 750.00 nm
- **Analytical Balance** Model: CX -220, Citizen
- **Sonicator** Model: TRANS-O-SONIC; D-compect., Capacity: 2 Lit.
- **pH meter** Model: digital pH meter, Analab scientific instrumentation pvt. ltd.
- **Water bath** Model: Water bath with digital temp. controller, EIE instrument pvt. ltd.
- **Hot air oven** Model: Hot air oven with digital temp. controller, EIE instrument pvt. ltd.
- **Microcentrifuge** Model : Cooling centrifuge (C-24BL), Manufacturer REMI.

4.4.2 MATERIALS

- API Memantine Hydrochloride (MEM) gifted sample from INTAS pharmaceuticals Pvt. Ltd.
- API Donepezil Hydrochloride (DH) gifted sample from INTAS pharmaceuticals Pvt. Ltd.
- OPA (AR Grade, Sisco Research Laboratories Pvt Ltd.)
- β – mercaptoethanol (for molecular biology, AR Grade, Sisco Research Laboratories Pvt Ltd.)
- Boric acid (AR Grade, S.D.Fine Chemicals Ltd., Bombay, India)
- Potassium chloride (AR Grade, S.D.Fine Chemicals Ltd., Bombay, India)
- Sodium hydroxide (AR Grade, S.D.Fine Chemicals Ltd., Bombay, India)
- Trichloroacetic acid (AR Grade, S.D.Fine Chemicals Ltd., Bombay, India)
- Perchloric acid (AR Grade, S.D.Fine Chemicals Ltd., Bombay, India)
- n- Hexane (AR Grade, S.D.Fine Chemicals Ltd., Bombay, India)
- Iso-propyl alcohol (AR Grade, S.D.Fine Chemicals Ltd., Bombay, India)
- Blank human plasma (kindly gifted by PRATHMA BLOOD BANK, Ahmedabad)

4.4.3 METHOD

4.4.3.1 PREAPARATION OF SOLUTIONS

4.4.3.1.1 Preparation of standard stock solution of MEM and DH

MEM (25 mg) and DH (25mg) were accurately weighed and transferred to two separate 25 mL volumetric flasks. Dissolved in 10 mL Double distilled water, sonicated for 10 min and volume made up to mark with double distilled water to obtain standard stock solution having concentration 1000 µg/mL each. 5 mL aliquot from this solution transferred to 50 mL volumetric flask and volume made up with double distilled water to obtain working standard stock solution of 100 µg/mL**.**

4.4.3.1.2 Preparation of Borate buffer pH (9.6)

Boric acid (3.09 gm) and Potassium Chloride (3.73 gm) were accurately weighed and transferred to 250 mL volumetric flask. Volume was made up with distilled water. Then solution was transferred to 1000 mL beaker and pH of solution adjusted to 9.6 by addition of 0.2 M of Sodium Hydroxide solution. After pH adjustment volume was made up 1000mL with distilled water.

4.4.3.1.3 Preparation of derivatization reagent

OPA (50 mg) was taken in 25 mL amber colored volumetric flask, 5 mL methanol was added, sonicated for 5 minutes. β-mercaptoethanol (20 µL) was added and finally volume was made up to mark with borate buffer pH 9.6 and store at temperature below 10^0C

4.4.3.2 EXTRACTION PROCEDURE

500µl of plasma sample was taken in 2mL microcentrifuge tube then 50 µl of drug solution was spiked and 500 µl of 5% TCA solution was added. Centrifuged for 3 min at 5000 rpm, then supernatant was transferred in another 2 mL microcentrifuged tube and 100 µl of 0.1 N NaOH solution to make supernatant alkaline. To this microcentrifuge tube 1 mL of 5 % IPA in n-Hexane solution was added and centrifuged for 3 min at 8000 rpm and upper organic phase was separated and remaining aqueous phase was again centrifuged with 1 mL 5 % IPA in n- Hexane solution and upper organic phase was separated and both organic phase were taken in 10 mL volumetric flask and evaporated to dryness. Then reconstituted with borate buffer to made up volume 10 mL and analyzed by spectroflurometer.

4.4.3.3 Derivatization of MEM:

After extraction of MEM from plasma as per section, in dried volumetric flask containing MEM added 1 mL borate buffer (1 mL) and 0.1 mL of derivatization solution was added and

kept at water bath for 10 min at 70^0c. After 10 minutes volume was made with borate buffer and solution were kept at room temp. for next 10 minutes. After that solution were scanned and this can be done within 10 minutes.

4.4.3.4 OPTIMIZATION OF EXPERIMENTAL CONDITIONS

- Extraction solvent
- Sensitivity

4.4.3.4 SELECTION OF ANALYTICAL WAVELENGTH

From the working standard solutions 1 µg/mL solutions were prepared by appropriate dilution of solvent and scanned in the synchronous mode with delta value 65 (medium sensitivity mode) from 220 to 680 nm. From the overlain synchronous spectra of these drugs, wavelengths 389nm selected for estimation of DH and 420 nm for MEM were selected for analysis.

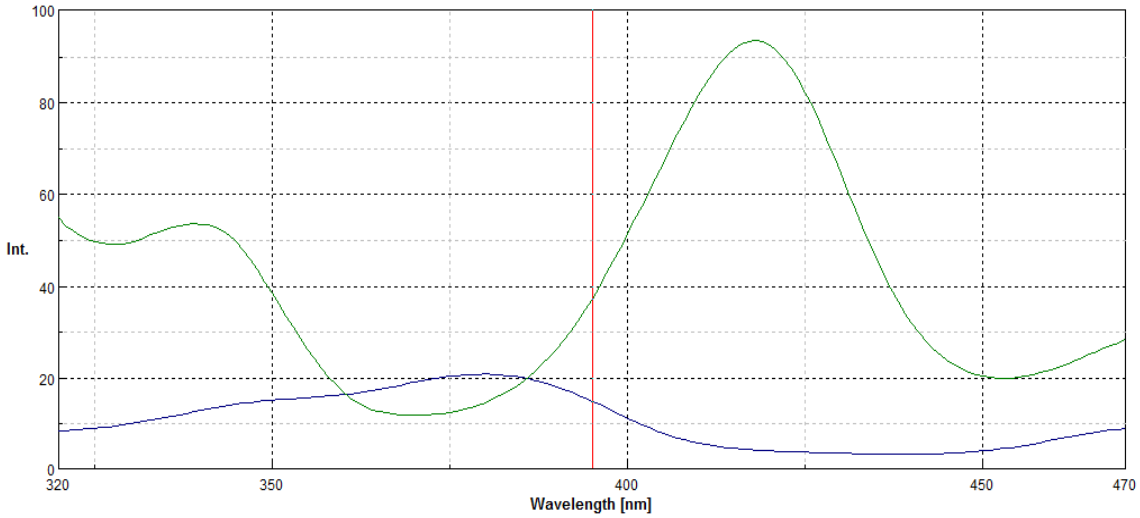

Fig 4.31:Overlay synchronous spectra (medium sensitivity) of DH 25 ng/mL and MEM 100 ng/mL in borate buffer (pH 9.6)

4.4.3.4 METHOD VALIDATION:

4.4.3.4.1 Linearity

Preparation of calibration curve

FOR MEM

For preparation of calibration curve of MEM, 500 µg of blank plasma was spiked with 50 µg of drug solutions of concentration of 10 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL,50 µg/mL, 100µg/mL, to get the final concentration of 50 ng/mL, 100 ng/mL, 150 ng/mL, 200 ng/mL, 250

ng/mL, 300 ng/mL respectively. Then extraction was performed as per section 4.4.3.2 and further MEM was derivatized as per section 4.4.3.3, then solutions were scanned in synchronous mode.

FOR DH

For preparation of calibration curve of DH, 500 µg of blank plasma was spiked with 50 µL of drug solutions of concentration of 1 µg/mL, 5 µg/mL, 9 µg/mL, 13 µg/mL, 17µg/mL, 21µg/mL, 25 µg/mL and 29 µg/mL to get the final concentration of 5 ng/mL, 25 ng/mL, 45 ng/mL, 65 ng/mL, 85 ng/mL, 105 ng/mL, 125 ng/mL, 145 ng/mL and 165 ng/mL respectively. Then extraction was performed as per section 4.4.3.2 and solutions were scanned in synchronous mode.

4.4.3.4.2 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).

4.4.3.4.2 Intraday and Interday precision:

FOR MEM

Intra-day and Inter-day precision was determined by measuring the intensity MEM three times within a day and on three different days, respectively. For intraday and interday precisions studies 100, 200 and 300 ng/mL were selected. 500 µL of blank plasma was spiked with 50 µL of drug solution of concentration of 20 µg/mL, 40 µg/mL, 60µg/mL to get the final concentration of 25 ng/mL, 65 ng/mL and 105 ng/mL respectively. Then extraction was performed as per section4.3.3.2. Then extraction was performed as per section 4.4.3.2 and further MEM was derivatized as per section 4.4.3.3 and solutions were scanned in synchronous mode.

FOR DH

Intra-day and Inter-day precision was determined by measuring the intensity of DH three times within a day and on three different days, respectively. For intraday and interday precision Intra-day precision 25, 65, 105 ng/mL were selected. 500 µL of blank plasma was spiked with 50 µL of drug solution of concentration of 5 µg/mL, 13 µg/mL, 21µg/mL to get the final concentration of 25 ng/mL, 65 ng/mL and 105 ng/mL respectively. Then extraction was performed as per section 4.4.3.2 and solutions were scanned in synchronous mode.

4.4.3.4.3 Recovery:

The % recovery of Drug was determined by comparing the peak intensity of pure drug with those obtained after extraction from plasma.

For MEM recovery studies, 500 µL of blank plasma was spiked with 50 µL of drug solutions of concentration of 10 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL,50 µg/mL, 100µg/mL, to get the final concentration of 50 ng/mL, 100 ng/mL, 150 ng/mL, 200 ng/mL, 250 ng/mL, 300 ng/mL respectively. Then extraction was performed as per section 4.3.3.2 and further MEM was derivatized as per section 4.4.3.3.The response of these solution was compared with pure drug solutions reponse which are prepared from working standard solution and derivatized as per section 4.4.3.3.

For DH recovery studies, 500 µL of blank plasma was spiked with 50 µL of drug solutions of concentration of 1 µg/mL, 5 µg/mL, 9 µg/mL, 13 µg/mL, 17µg/mL, 21µg/mL, 25 µg/mL and 29 µg/mL to get the final concentration of 5 ng/mL, 25 ng/mL, 45 ng/mL, 65 ng/mL, 85 ng/mL, 105 ng/mL, 125 ng/mL, 145 ng/mL and 165 ng/mL respectively. Then extraction was performed as per section 4.4.3.2. The response of these solution was compared with pure drug solutions reponse which were prepared from working standard solution.

Chapter 5
Comparision and Conclusion

Comparison

Comparison of developed UV-spectrophotometric and spectrofluorimetric methods was performed by applying unpaired t- test using SPSS software to assay Results (table 47). The results are shown in table 48.

Table 5.1: Assay Results of the Proposed Methods

Drug	% Assay	
	UV method	Spectrofluori method
MEM	99.56	98.11
	99.31	98.76
	98.23	98.02
DH	98.46	99.21
	97.87	98.73
	98.96	99.41

Table 5.2: Results of unpaired t-test

	MEM		DH	
Parameters	UV method	Spectrofluori method	UV method	Spectrofluori method
Mean % Assay	99.03	98.3	98.43	99.12
Variance	0.4081	0.2331	0.3150	0.2018
Observations	3	3	3	3
Degree of freedom	2	2	2	2
P value	0.1921		0.1403	

Output of unpaired t-test at 95% confidence interval, shows that there is no significant difference with respect to % assay between the proposed UV-spectrophotometric and spectrofluorimetric methods.

P value for both the drugs obtained in unpaired t-test for comparison between UV spectrophotometric and spectrofluorimetric method was found to be more than 0.05 so it indicates that there is not significant difference in both the methods.

Conclusion

The developed and validated UV spectrophotometric and spectrofluorimetric methods are simple, rapid, accurate, sensitive, precise, and robust for determination of MEM and DH in combined tablet dosage form. The excipients usually present in the pharmaceutical formulation did not interfere with determination of MEM and DH. Any of the developed method can be successfully used for routine quality control of MEM and DH in their combined dosage form. Assay results of tablet obtained by these two methods were compared using t- test, which reveals that there is no significant difference between the proposed methods.

Spectrofluorimetric method has more sensitivity as compare to UV spectrophotometric method but UV spectrophotometric method has advantage of stability of derivative as compared to spectroflurimetric method.

Hence, it was concluded that both UV- spectrophotometric and spetrofluorimetric methods do not differ significantly.

Spiked human plasma study of MEM and DH by spectrofluometric method results in acceptable linearity and % recovery, hence it can be applied for estimation of both the drugs in plasma.

Chapter 6
Summary

UV spectrophotometric, spectrofluorimetric methods were developed and validated for the simultaneous estimation of MEM and DH in bulk and pharmaceutical formulation. Spectrofluorimetric method was further applied for estimation of MEM and DH in human plasma.

OPA-βmercaptoethanol derivatization of MEM was applied in both UV spectrophotometric and spectrofluorimetric methods because of absence of chromophoric group in MEM. Derivatization process includes reaction of MEM with OPA in presence of βmercaptoethanol under alkaline medium results in OPA-MEM derivative having λ_{max} 337 nm in methanol for UV method and λ_{exci} 338 nm, 430 nm λ_{emi} in borate buffer for spectrofluorimetric method, respectively.

Derivatization method was optimized in terms of OPA conc. (0.2% w/v in derivatization reagent solution), β-mercaptoethanol conc. (20µL in 25ml derivatization reagent solution), reaction temperature (70^0C), reaction time (10 minutes) and pH of Borate buffer (9.6) used in preparation of derivatization reagent solution.

UV Spectrophotometric method includes Simultaneous equation method that involves measurement of absorbances at two wavelengths i.e. at 337 nm (λ_{max} of MEM) and 312.6 nm (λ_{max} of DH) in methanol. Linearity range was observed in the concentration range 5-80 µg/mL with mean recovery of 98.91% ± 1.23 and 99.87 %± 0.68, for MEM and DH respectively. The correlation coefficients for MEM and DH were found to be 0.9984 and 0.9987, respectively. LOD and LOQ were found to be 1.220 and 3.698 µg/mL for MEM, respectively and 0.775 and 2.347 µg/mL for DH, respectively. The R.S.D. values for precision studies were found to be less than 2 for both the drugs.

Spectrofluorimetric method includes synchronous spectrum method using delta value 65 (low sensitivity mode) that involves measurement of fluorescence intensity at two wavelengths in synchronous spectra of drugs i.e. at 450 nm for MEM (zero crossing point for DH) and 361 nm (zero crossing point for MEM) in borate buffer. Linearity range was observed in the concentration range 1-30 µg/mL with mean recovery of 98.01% ± 0.57 and 98.82 %± 0.68 for MEM and DH, respectively. The correlation coefficients for MEM and DH were found to be 0.9986 and 0.9983, respectively. LOD and LOQ were found to be 0.228 and 0.691 µg/mL for

MEM, respectively and 0.135 and 0.410 μg/mL for DH, respectively. The R.S.D. values for precision studies were found to be less than 2 for both the drugs.

Assay results of the marketed formulation obtained from both methods were satisfactory.

Both the methods were compared using t- test at the 95% confidence interval, which reveals that there is no significant difference with respect to accuracy and precision between the proposed methods.

Estimation of MEM and DH in human plasma was done by spiked human plasma studies. Extraction was carried out using 5% TCA for protein precipitation followed by liquid-liquid extraction with 5% IPA in n-Hexane. Under spectrofluorimetric conditions delta value 65 applied in synchronous mode (medium sensitivity mode) and wavelength used in analysis were 389nm and 420 nm for MEM and DH, respectively. Linearity range was observed in the concentration range 5-105 ng/mL and 50-300 ng/mL for DH and MEM respectively. The correlation coefficients for DH and MEM were found to be 0.9965 and 0.9951, respectively. Percentage recovery was found to be 74.43%-83.33% and 77.85%-83.68% for DH and MEM, respectively. The R.S.D. values for precision studies were found to be less than 10 for both the drugs.

Chapter 7
Future scope

Future Scope

This method can be further extended for the estimation of both drugs from the plasma.

Apart from this different new analytical techniques like Raman spectroscopy, HPTLC, RP-HPLC, Near IR Spectrometry and Super critical fluid chromatography (SFC) can be developed for this combination

Stability indicating different techniques can be developed for the same.

Chapter 8
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