"SIMULTANEOUS ESTIMATION OF GABAPENTIN AND NORTRIPTYLINE HYDROCHLORIDE IN THEIR COMBINED DOSAGE FORM BY UV-SPECTROPHOTOMETRIC AND SPECTROFLUORIMETRIC METHODS USING OPA-β-MERCAPTOETHANOL DERIVATIZATION"

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IN

PHARMACEUTICAL ANALYSIS

BY

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CERTIFICATE

This is to certify that the dissertation work entitled "Simultaneous estimation of gabapentin and nortriptyline hydrochloride in their combined dosage form by UV-spectrophotometric and spectrofluorimetric methods using OPA-β-mercaptoethanol derivatization" submitted by **Mr. Ankit Shah** *with Regn. No. (12MPH301) 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 "Simultaneous estimation of gabapentin and nortriptyline hydrochloride in their combined dosage form by UV-spectrophotometric and spectrofluorimetric methods 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, 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	
GABA	Gabapentin
NTH	Nortriptyline 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	Litre
L/h	Liter per hour
Mg	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	
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

Accurate, Sensitive and reproducible UV-spectrophotometric and Spectrofluorimetric methods were developed for the simultaneous estimation estimation of Gabapentin (GABA) and Nortriptyline hydrochloride (NTH) in their combined dosage form. This paper describes validated UV spectrophotometric, spectrofluorimetric methods for the simultaneous estimation of Gabapentin (GABA) and Nortriptyline hydrochloride (NTH) in bulk and combined tablet dosage form. The proposed methods include derivatization of GABA with OPA-βmercaptoethanol due to lack of chromophoric group. UV Spectrophotometric method includes Absorbance corrected method that involves measurement of absorbance at two wavelengths i.e. at 335 nm (λmax of GABA) and 241 nm (λmax of NTH) in methanol. Linearity range was observed in the concentration range 160-560 µg/mL with mean recovery of 100.96 % ± 0.6769 for GABA and 4-14 µg/mL with mean recovery of 99.73 % ± 0.5502 for NTH respectively. The correlation coefficients for GABA and NTH were found to be 0.9994 and 0.9999, respectively. LOD and LOQ were found to be 2.390 and 7.241 µg/mL for GABA 0.010 and 0.030 µg/mL for NTH, respectively. Spectrofluorimetric method includes synchronous simultaneous equation method using delta value 65 (medium sensitivity mode) that involves measurement of fluorescence intensity at two wavelengths in synchronous spectra of drugs i.e. at 417 nm for GABA and 377 nm for NTH in borate buffer. Linearity range was observed in the concentration range 10-90 µg/mL with mean recovery of 100.73 % ± 0.7422 for GABA and 0.25-2.25 µg/mL with mean recovery of 100.08 % ± 0.1744 for NTH, respectively. The correlation coefficients for GABA and NTH were found to be 0.9989 and 0.9984, respectively. LOD and LOQ were found to be 0.596 and 1.806 µg/mL for GABA and 0.060 and 0.181 µg/mL for NTH, respectively. The validation was done by ICH guidelines and successfully applied to marketed tablet formulation.

Keywords – Gabapentin, Nortriptyline hydrochloride, Synchronous Spectrofluorimetry, UV Spectrophotometry, Tablet dosage form.

Chapter-1
Introduction

1.1 INTRODUCTION TO DISEASE [1-5]

Depression is a state of low mood and aversion to activity that can affect a person's thoughts, behavior, feelings and physical well-being .Depressed people might feel sad, anxious, empty, hopeless, helpless, irritable, worthless, guilty, , or restless. They may lose interest in activities that once were pleasurable; experience loss of appetite or overeating, have problems in remembering details ,concentrating, or making decisions; and may contemplate or attempt suicide. Insomnia, excessive sleeping, fatigue, loss of energy, or pains, aches or digestive problems that are resistant to treatment may be present.

Depressive disorders are mood disorders , come in different forms, just as other illnesses do , such as cardiac diseases and diabetes. Three most common types of depressive disorders are discussed below. However, remember that within each of these types, there are variations in the number, timing, severity, and persistence of signs. There are also differences in how individuals experience depression based on age.

Major depression

Major depression is characterized by a combination of symptoms that last for at least two weeks in a row, including sad and/or irritable mood , that interfere with the efficiency to work, sleep, eat, and enjoy once-pleasurable activities. Difficulties in sleeping or eating could take the form of excessive or insufficient of either behavior. Disabling episodes of depression can occur once, twice, or several times in a lifespan.

Dysthymia

Dysthymia is a less severe and usually more long-lasting type of depression compared to major depression. It involves long-term (chronic) symptoms that do not disable but yet prevent the affected person from functioning at "full steam" or from feeling good. Sometimes, people with dysthymia also experience episodes of major depression. This combination of the two types of depression is called as double-depression.

Bipolar disorder (manic depression)

Another type of depression is bipolar disorder, which involves a group of mood disorders that were formerly called manic-depressive illness or manic depression. These conditions shows a

particular pattern of inheritance. Not nearly as common as the other types of depressive disorders, bipolar disorders involve cycles of moods that includes at least one episode of mania or hypomania and can include episodes of depression as well. Bipolar disorders are often chronic and recurring. Sometimes, the mood switches are dramatic and rapid, but more over they are gradual.

When person in the depressed cycle, can experience any or all of the symptoms of a depressive disorder. When in the manic cycle, any or all of the symptoms listed further under mania may be experienced. Mania often affects social behavior, thinking and judgment in ways that cause serious problem and embarrassments. For example, indiscriminate or otherwise unsafe sexual practices or unwise business or financial decisions may be made when a person is in a manic phase.

A significant variant of the bipolar disorders is termed as bipolar II disorder. (The usual form of bipolar disorder is referred to as bipolar I disorder.) Bipolar II disorder is a syndrome in which the affected person has repeated depressive episodes affected by what is called hypomania (mini-highs). These euphoric states in bipolar II can not fully meet the criteria for the complete manic episodes that occur in bipolar I.

Symptoms of depression and mania

Not everyone who is depressed or manic experiences every symptom. Some people may experience a few symptoms and some many symptoms. The severity of symptoms also varies with individuals. Less severe symptoms that precede the more debilitating symptoms are called as warning signs.

Depression symptoms of major depression or manic depression[6]

- Persistently unhappy, anxious, angry, irritable, or "empty" mood.
- Feelings of hopelessness or nervousness.
- Feelings of worthlessness, helplessness and excessive guilt.
- Loss of interest or pleasure in hobbies and activities that were once enjoyed, including sex , social isolation (the sufferer avoids interactions with family, relatives or friends).
- Insomnia characterized as early-morning awakening, or oversleeping.

- Decreased appetite and/or weight loss, or overeating and/or weight gain Fatigue, decreased energy, being "slowed down".
- Crying spells.
- Thoughts of death or suicide, suicide attempts.
- Restlessness, irritability.
- Difficulty concentrating, remembering, or making decisions
- Persistent physical symptoms that do not respond to treatment, such asheadaches, digestive disorders, severe chronic pain.

Mania symptoms of manic depression

- Inappropriate irritability or anger
- Inappropriate elation
- Severe insomnia or decreased need to sleep
- Grandiose notions, like admiring special powers or importance
- Increased talking speed and/or volume
- Disconnected thoughts and speech
- Racing thoughts
- Severely increased sexual desire and/or activity
- Markedly increased energy
- Poor judgment
- Inappropriate social behavior.

There are many medications available to treat **depression**.

Different types of antidepressant agents include:

- Tricyclic antidepressants **(TCAs)**
- Selective serotonin reuptake inhibitors **(SSRIs)**
- Serotonin-norepinephrine reuptake inhibitors **(SNRIs)**
- Monoamine oxidase inhibitors **(MAOIs)**
- Miscellaneous other antidepressants.

Tricyclic Anti-Depressants (TCAs)

The TCAs and related drugs inhibit active reuptake of biogenic amines such as NA and 5-HT into their respective neurons and thus they potentiate them. They, however , differ makedly in their selectivity and potency for different amines in neurons.

Reuptake inhibition results in increased concentration of the amines in the synaptic cleft in the CNS and periphery which is associated with antidepressant action[7].

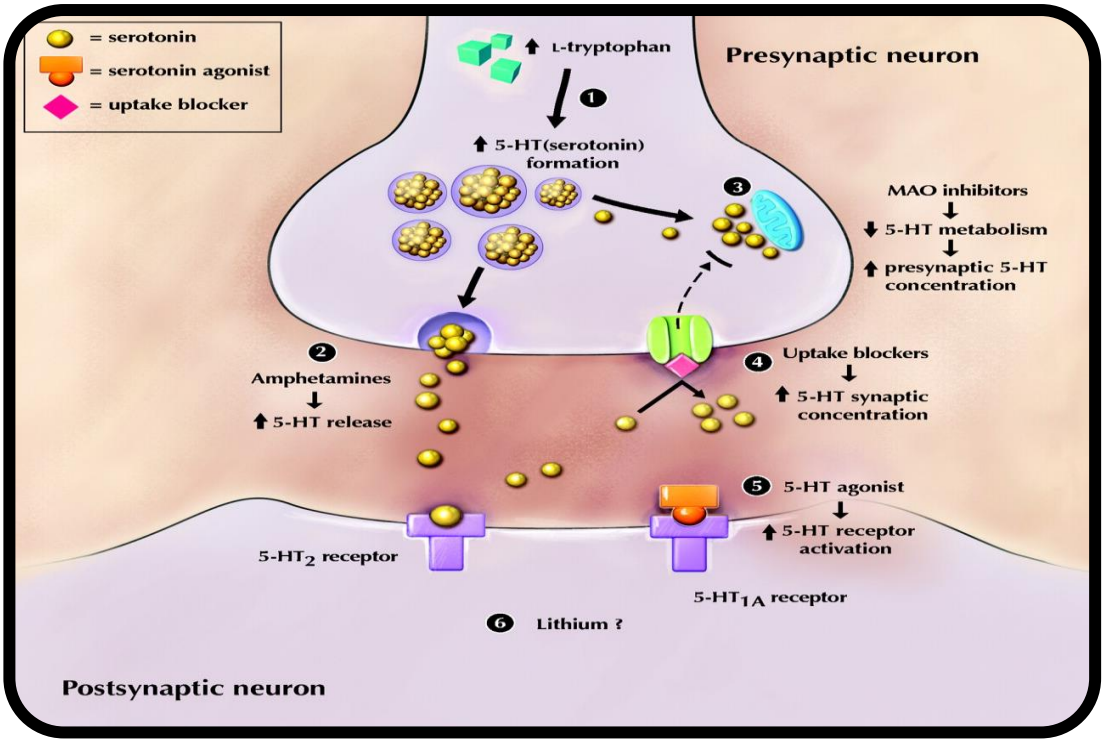


Fig. 1.1 Antidepressant agents Pathways

1.2 DRUG PROFILE

1.2.1 GABAPENTIN[8-10] :

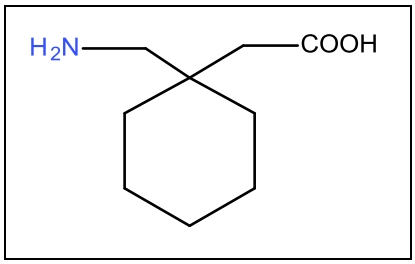


Fig.1.2 Structure of GABA

IUPAC Name : 2-[1-(aminomethyl)cyclohexyl]aceticacid

Molecular Formula : $C_9H_{17}NO_2$

Molecular Weight : 171.2

CAS No. : 60142-96-3

Category : Antianxiety agent, Anticonvulsant, Antiparkinson agent, Analgesic

Official Status : Official in USP32(NF27) and Merk index

Drug Approval : Approved by USFDA in 1994.

Physicochemical Properties :

1. Appearance : It is a white to off-white crystalline solid.

2. Solubility : Freelysoluble in water , HCl , NaOH ,Methanol and Glacial Acetic acid.

3. Melting Point : 162^0-166^0C

4. Dissociation Constant : pKa (strongest acidic) = 4.63
pKa (strongest basic) = 9.91

5. Storage : Store protected from light and moisture, at a temperature not exceeding 30°C.

Table 1.1 : Pharmacokinetic parameters of GABA

Bioavailabilty and dose Proportionality	Bioavailability ranges from approximately 60% for a 900 mg dose per day to approximately 27% for a 4800 milligram dose per day.
$T_{1/2}$	5 to 7 hrs
Protein binding	Less than 3%
Metabolism and Elimination	Not appreciably metabolized, Renal Eliminatation occur.
Drug- Drug interaction	There is no interaction between gabapentin and phenytoin, valproic acid, carbamazepine or phenobarbitone. Gabapentin steady-state pharmacokinetics are similar for healthy subjects and patients with epilepsy receiving anti-epileptic agents.
Adverse effect	side effects in adult patients include dizziness, fatigue, weight gain, drowsiness, and peripheral edema, increased risk of suicidal acts or violent deaths.

Mechanism of Action : Gabapentin reacts with voltage-sensitive calcium channels in cortical neurons. Gabapentin increases the synaptic concentration of GABA, increases GABA responses at non-synaptic sites in neuronal tissues, and reduces the release of mono-amine neurotransmitters. Studies have shown that the antihyperalgesic and antiallodynic effects of gabapentin are mediated by the descending noradrenergic system receptors, gives the activation of spinal alpha2-adrenergic receptors. Gabapentin does not interact with GABA receptors, and is not metabolized to a GABA agonist or to GABA agonist or to GABA, and does not inhibit GABA uptake or degradation.

1.2.2 NORTRIPTYLINE HYDROCHLORIDE[11-13] :

- Structural Formula :

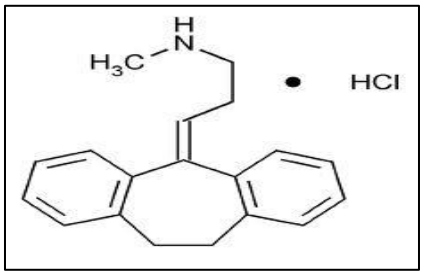


Fig. 1.3 Structure of Nortriptyline Hydrochloride

- IUPAC Name : 1-propanamine,3-(10,11-dihydro-5*H*-dibenzo -[*a*,*d*]cyclohepten-5-ylidene)-*N*-methyl-,hydrochloride.
- Molecular Formula : $C_{19}H_{21}N.HCl$
- Molecular Weight : 299.84
- CAS No. : 894-71-3
- Category : Antidepressant.
- Official Status : Official in British Pharmacopeia 2014, USP32 (NF27) , Japanese Pharmacopoeia XIV and Merk index.
- Drug Approval : Approved by USFDA in 23 july, 1993.

- **Physicochemical Properties :**

1. Appearance : White to yellowish white , crystalline powder.

2. Solubility : Freely Soluble in acetic acid(100) and in Chloroform , soluble in ethanol(95%) , sparingly soluble in water.

3. Melting Point : 215^0-220^0C

4. Dissociation constant : pKa (strongest acidic) = 5.24

pKa (strongest basic) = 10.47

5. Storage : Store at 15°c to 30°C in tightly closed container, protected from light and moisture.

Table 1.2 : Pharmacokinetic parameters of NTH

Bioavailabilty and dose Proportionality	High bioavailability and For depression low starting doses are used, increasing as necessary to 75–100mg. Maximum daily dosage is 150 mg.
$T_{1/2}$	16 and 90 hours
Protein binding	Highly protein-bound in plasma and tissues.
Metabolism and Elimination	Metabolised by CYP450 2D6 enzyme, Approximately one-third of a single orally administered dose is excreted in urine within 24 hours. Small amounts are excreted in feces via biliary elimination.
Adverse effect	Dry mouth, sedation, constipation, and increased appetite, mild blurred vision, tinnitus, often euphoria and mania.

- Mechanism of Action :

Nortriptyline Hydrochloride either inhibits the reuptake of the neurotransmitter serotonin at the neuronal membrane or acts at beta-adrenergic receptors. Tricyclic antidepressants do not inhibit monoamine oxidase nor they affect dopamine reuptake. They inhibits activity of histamine, 5-HT, and acetylcholine. It also increases pressor effect of norepinephrine but blocks pressor response of phenethylamine. It also interferes with transport, release and storage of catecholamines. The principal effects of the tricyclic antidepressants on the function of the autonomic nervous system are accepted to result from inhibition of norepinephrine transport into adrenergic nerve terminals and from antagonism of muscarinic cholinergic and alpha1-adrenergic responses to the autonomic neurotransmitters.

1.3 RATIONALE OF DRUG COMBINATION

Gabapentin is an antiepileptic drug which is a structural analogue of neurotransmitter γ-aminobutyric acid (GABA). It was firstly developed for the treatment of epilepsy, and currently it is also used to relieve neuropathic pain. It is orally administered and its bio-availability is rapid. It have less protein binding (<3%) and half-life is 5-7 hours. The pharmacology of Gabapentin involves binding with the voltage-gated calcium channels and reduces the release of several neurotransmitters which would reduce neuronal excitability and seizures.

Nortriptyline hydrochloride is a predominantly NA+5HT reuptake inhibitor tricyclic antidepressant drug, widely used in the treatment of unipolar depression, since it is a non-selective serotonine uptake inhibitor.

Study finds that the combination of gabapentin and nortriptyline hydrochloride decreases depression more than either medication alone in the treatment of neuropathic pain from diabetic polyneuropathy. The combination of gabapentin and nortriptyline hydrochloride is mostly useful in treatment of depression than single drug therapy[14].

1.4 INTRODUCTION TO DERIVATIZATION REACTION :

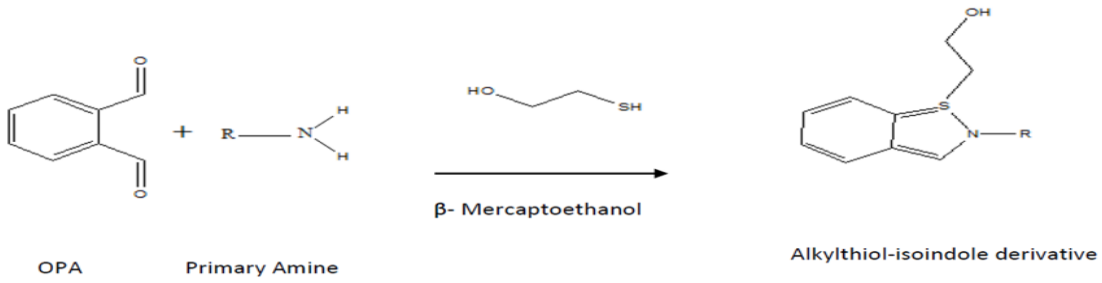


Fig.1.4 Derivatization Reaction

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 can shows λmax 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[15].

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.
- VALIDATION OF ANALYTICAL METHODS.

Method Development

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 only 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 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 should not be official in any pharmacopoeias.
- A proper analytical procedure for the drug should not be available in the literature due to patent regulations.
- Analytical methods should 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 should not be available.
- Analytical methods for a drug in combination with other drugs should not be available[16].

1.5.1 UV-VIS Spectrophotometric Method

Ultraviolet-visible spectroscopy or ultraviolet–visible spectroscopy (UV-Vis) involves the spectroscopy of photons in the UV-visible region. It uses light in the visible and near [near ultraviolet (UV) and near infrared (NIR)] ranges. The absorption in the visible ranges directly affect the color of the chemicals and substances involved. In the region of the electromagnetic spectrum, molecules rapidly 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 always measures transitions from the ground state to the excited state.

The spectrophotometric assay of the drugs rarely involves the measurement of absorbance of samples containing only one absorbing component. The pharmaceutical analyst frequently faces 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 can be determined.

A number of modifications to the simple spectrophotometric procedure are available to the chemist, 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 mean to be 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 drug components,
- The measured absorbance is the difference between the total absorbance of the sample solution in the sample cell and that of the solution in the reference cell.

There are various UV-spectrophotometric methods are available for quantification of multicomponent drug samples. Following methods can be used ,

- Simultaneous equation method
- Absorbance correction method
- Derivative spectrophotometric method
- Absorbance ratio method (Q-Absorbance method)
- Difference Spectrophotometry
- Geometric correction method
- Orthogonal polynomial method
- Least square approximation
- Dual wavelength spectrometry

Absorbance Correction method

If the absorptivity, identity and concentration of the absorbing interferents are known , it can be possible to calculate their contribution to the total absorbance of the mixture. The concentration of the absorbing substance of interest is then calculated from the corrected absorbance (total absorbance-absorbance of interfering substances) in the same way.

Example,

The λ_{max} of ephedrine hydrochloride is 257 nm and 279 nm for chlorocresol and the A(1%,1cm) values in 0.1 M hydrochloric acid solution are ,

ephedrine hydrochloride at 257 nm : 9.0 , chlorocresol at 257 nm : 20.0 , ephedrine hydrochloride at 279 nm : 0 , chlorocresol at 279 nm : 105.0

Calculate the conc. of chlorocresol and ephedrine hydrochloride in a batch of ephedrine hydrochloride Injection , diluted 1 to 20 with water , giving the following absorbance values in 1 cm cells. A_{279}=0.425 and A_{257}=0.975.

Now,

ephedrine hydrochloride is not absorbing at 279 , calculating the conc. of chlorocresol from the A_{279} of the diluted formulation,

0.425 = 105 x 1 x c

C = 0.00405 g/100 ml

Conc. of chlorocresol in the injection

=0.00405 x 20

=0.081 g/100 ml

=0.81 mg/ml

Now, calculating the absorbance of chlorocresol at 257 nm in the diluted injection

A = 20 x 1 x 0.00405

A = 0.081

Now, calculating the conc. of ephedrine hydrochloride from corrected absorbance at 257 nm,

Corrected absorbance at 257 nm = 0.975 – 0.081

$$A_{257} = 0.891$$

0.891 = 9.0 x 1 x c

C = 0.0990 g/100 ml

Now, Conc. of ephedrine hydrochloride in injection

= 0.0990 x 20

= 1.98 g/100 ml

= 19.8 mg/ml

Thus, Absorbance corrected method can be introduce in the calculation of the concentrations of two drugs.

Simultaneous Equation Method (Vierodt's method)

Simultaneous equation method is a quantitative method, mostly used when two absorbing species in the solution have an overlapping spectra. If a sample contains two absorbing drugs (X and Y) each of which absorb at the λmax of the other (as shown in figure 1.6 as λ_1 and λ_2), it can be possible to determine both drugs by the technique of simultaneous equation (Vierodt's method).

The information's required are:

- The absorptivities of X at λ1 and λ2, ax1 and ax2 respectively
- The absorptivities of Y at λ1 andλ2, ay1 and ay2 respectively
- The absorbances of the diluted sample at λ1 and λ2, A1 and A2 respectively.
- The concentration of X and Y(Cx and Cy respectively) in the diluted samples.

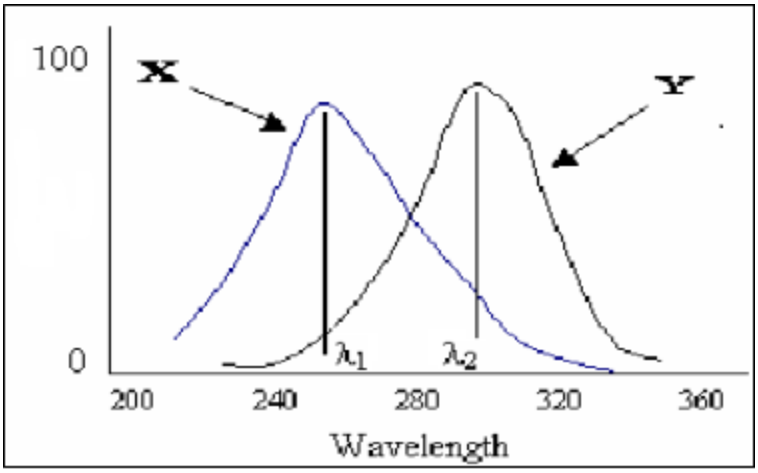


Fig. 1.5 Wavelength selections by simultaneous equation method

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

$$\mathbf{Cx} = \frac{\mathbf{A2\ X\ ay1 - A1\ X\ ay2}}{\mathbf{ax2\ X\ ay1 - \ ax1\ X\ ay2}} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \mathbf{1}$$

$$\mathbf{Cy} = \frac{\mathbf{A1\ X\ ax2 - A2\ X\ ax1}}{\mathbf{ax2\ X\ ay1 - \ ax1\ X\ ay2}} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \mathbf{2}$$

Here, A_1 and A_2 are absorbance of the mixture at λ_1 and λ_2 nm respectively; ax_1 and ax_2 are absorptivity values of Drug-X at λ_1 and λ_2 nm and ay_1 and ay_2 are absorptivity values of Drug-Y at the two wavelengths respectively[17,18].

1.5.2 Introduction to Spectrofluorimetric Method

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

When light of an appropriate wavelength is absorbed by a molecule (i.e.,excitation) the electronic state of the molecule alters 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 1.5). Once the molecule is in this excited state, relaxation may occur via several processes, Fluorescence is one of these processes and results in the emission of light.

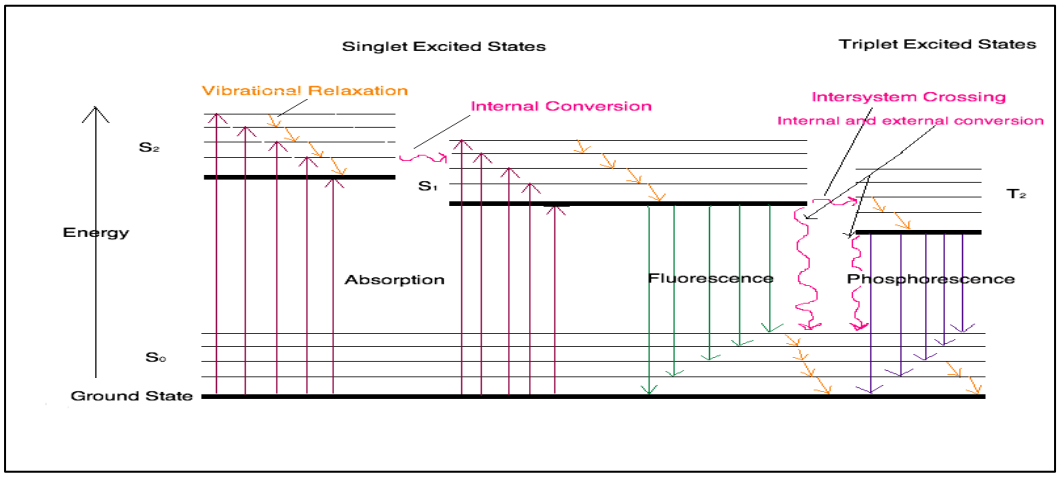


Fig. 1.6 Electronic transition energy level diagram

Following absorption reaction, a number of vibrational levels of the 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 shown in Figure 1.5, vibrational coupling can occur between the two states. Molecules in the single 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 state with emission of light. Because this is a classically forbidden transition, the triplet state have 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 lights. Fluorescence has short life time (~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 may be written as :

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

Where, Efluor is the energy of the emitted light, Eabs is the energy of the light absorbed by themolecule during excitation, and Evib is the energy lost by the molecules from vibrational relaxation and Esolv.relax term rises 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 (10-12 sec) relative to the average lifetime of the lowest excited singlet state (10-8 sec) and therefore competes effectively with fluorescence in the most of the molecules.

Other processes, which can compete with fluorescence, are excited state isomerization, photoionization, photodissociation and acid-base equilibria. Fluorescence intensity can 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 may 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 can 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 may be improved by applying total luminescence or synchronous scanning fluorescence techniques. Synchronous fluorescence (SF) was introduced in the 1980s and it 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 emission wavelengths at the same velocity (synchronously) and consequently keeping a constant difference or increment between the wavelength of the emission and that of excitation of molecule.

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 may 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 the other scan modes.

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 hampered 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 the fluorescent signals from the 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 may 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 the reaction mechanisms and thermodynamic parameters by spectrofluorimetry.

In addition , the deeper knowledge reported every year about micellar media and its application to the recent 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 important topic in SF, due to its great specificity in the study of interfacial properties. Finally, SF has recently been incorporated into the 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 lastly ultraviolet (UV) spectrophotometry.

Sometimes, SF provides even better results than those from GC-MS or infrared (IR) spectroscopy, although results and conclusions seem to depend on the matrix studied. Thus, SF have 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 the 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 the quantitative determinations, especially in very concentrated and complex samples[20,21].

Simultaneous equation method can also be used for the quantitative estimation of two components. The method described under UV- spectrophotometric method can be applied same for the fluorimetric determination.

1.6 ANALYTICAL MEHOD VALIDATION[22]

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.

Objective of Validation :

The objective of validation of analytical procedure is to demonstrate that it is suitable for its intended purpose. Any developed method may be affected by variables like different elapsed assay times, different days, batches of reagents, instruments, equipments, environmental conditions like temperature, etc. 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 are reported.

For analytical method validation of pharmaceuticals, guidelines from the International Conference on Harmonization (ICH) , United States Food and Drug Administration (US FDA) , United States Pharmacopoeia (USP), and World Health Organization (WHO) provide a framework for performing such validations in a more efficient and prospective manner.

Green et al. have given a practical guide for analytical method validation, with a description of a set of minimum requirements for a method. Wegscheider et al. has published procedures for method validation with a special focus on calibration, recovery experiments, method comparison and investigation of ruggedness . Seno et al. have described how analytical methods are validated in a Japanese QC laboratory. The AOAC has developed a peer-verified methods validation program with detailed guidelines on exactly which parameters should be validated . Winslow and Meyer recommend the definition and application of the master plan for validating analytical methods. Breaux et al. have published a study on the analytical methods development and validation.

Validation Parameters :

I. 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, matrix, degradants etc.

II. 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 the analyte in the sample.

Linearity could 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.

III. 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.

IV. 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 can be considered at three levels: repeatability, intermediate precision and reproducibility.

1. Repeatability

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

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

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 the method precision study should not be more than 2.0% absolute.

3. Reproducibility

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

V. 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 which is found.

Several methods of determining the accuracy are available:

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

b) Comparison of the results of the proposed analytical procedure with those of a second well-characterized procedure, the accuracy which is mainly stated and/or defined.

c) Accuracy can 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%.

VI. Limit of Detection (LOD)

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 applied procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable,

1. Based on Visual Evaluation

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

2. Based on Signal-to-Noise

This approach may 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.

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 can be carried out in a variety of ways, for example ,

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.

3.2 Based on the Calibration Curve

A specific calibration curve could 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.

VII. Limit of Quantification (LOQ)

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,

1. Based on Visual Evaluation

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

2. Based on Signal-to-Noise Approach

This approach may 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.

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:

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.

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.

VIII. 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.

IX. 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 the % response of standard preparation after 24 hours and 48 hours storage against initial standard response. The assay value of sample and % response of standard calculated after 24 hours and 48 hours storage should be compared with the initial value and recorded.

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

Acceptance criteria:

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

Table 1.3 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

Chapter-2

Aim & Objective

Aim

- Several methods have been reported for the individual estimation of GABA and NTH and their combination with other drugs.

- Not a single UV Visible Spectrophotometric method and Spectrofluorimetric method is reported for estimation of GABA and NTH in combined dosage form.

- So, it was endeavored to develop and validate various analytical methods for simultaneous estimation of these drugs in combined dosage form.

Objective

The objectives of the work are :

- To develop UV spectrophotometric method for simultaneous estimation of GABA and NTH in combined tablet dosage form.
- To develop Spectrofluorimetric method for simultaneous estimation of GABA and NTH in combined tablet dosage form.
- Validate the developed methods as per ICH (Q2, R1) guideline.

Chapter-3

Literature Review

Table 3.1 Determination of GABA

Sr No.	Matrix	Method	Description	Reference No.
1)	Tablets	UV-Vis Spectrophotometric	Solvent: MeOH : Water(1:1) λ max : 240 nm. Linearity: 1-9 µg/mL	23
2)	Bulk, Capsule	UV-Vis Spectrophotometric	Solvent: Distilled Water λ max.: 210 nm. Linearity: 0.25 - 3.5 µg/mL	24
3)	Tablet	UV-Vis Spectrophotometric	Solvent: Ethanol: Water (1:1) λ max.: 265 nm. Linearity: 2-10 µg/mL	25
4)	Bulk, Tablet	UV-Vis Spectrophotometric	Method: standard curve method Solvent: water : 0.2% ninhydrin λ max.: 405 nm. Linearity: 50-300µg/ml	26

5)	Plasma, Capsule	UV-Vis Spectrophotometric	Method: Derivative Spectroscopy λ max.: 402 nm. Linearity: 10 – 90 µg/mL	27
6)	Bulk, Urine Samples	UV-Vis Spectrophotometric	Solvent: methanol – acetonitrile - potassium dihydrogen orthophosphate (pH5.2; 0.028 M) (25:10:65) λ max.: 210 nm. Linearity: 100–380 µg/mL	28
7)	Dosage forms	HPLC	Column: Nova-Pak®C184mm column (250mm 4.6 mm, Waters, Milford, MA, U.S.A.). Mobile Phase: acetonitrile-sodium dihydrogenphosphate (pH 2.5; 0.05 M) (70 : 30, v/v) Flow Rate: 1.5 mL/min. Wavelength: 360nm. Linearity: 10-500 µg/mL.	29

8)	Dosage formulations	HPLC	Column: Inertsil C18 column (250 mm × 4.6 mm) Mobile Phase: Methanol–water (80:20, v/v) Flow Rate: 1.2 mL/min. Wavelength: 458 and 521 nm for the excitation and emission Linearity: 5-50 µg/mL	30
9)	Bulk	HPLC	Column: Waters C18 5 µm column (150 mm ~ 4.6 mm) Mobile Phase: methanol – pot.dihydrogen orthophosphate (20:80, v/v) Flow Rate: 1.0 mL/min. Wavelength: 275nm. Linearity: 940-1060 µg/mL	31
10)	Formulations	HPLC	Column: Chromosil C18. 250×4.6mm Mobile Phase: Methanol: Acetonitrile: OPA 65:33:2 (v/v/v). Flow Rate: 1.0mL/min.	32

			Wavelength: 216 nm Linearity: 30-180 µg/mL	

Table-3.2 Determination of NTH

Sr No.	Matrix	Method	Description	Reference No.
1)	Tablets	UV-Spectrophotometric	Solvent: Methanol λ max.: 251nm. Linearity: 5.0–1350 µg/mL	33
2)	Plasma Samples	HPLC	Column: HP Hypersil silica column (150 x2.l-mm i.d., 5 um) Mobile Phase : ACN-0.1M ammonium acetate (94:6, v/v) Flow Rate: 0.4 mL/min. Wavelength: 240 nm Linearity: 0.01-1 µg/mL	34
3)	Plasma samples	HPLC	Column: Waters Spherisorb S5 ODS2 (4.6mm×250mm) Mobile Phase: ACN : water (85:15, v/v)	35

			Flow Rate: 1.0 mL/min. Wavelength: 260nm (excitation) and 310nm (emission). Linearity: 0.05-5 µg/mL	
4)	Bulk	HPLC	Column: 150mm × 4.0mm Discovery-C18 column from Supelco (Supelco Park, Bellefonte , PA,USA) Mobile Phase: acetonitrile and water (28:72, v/v) Flow Rate: 1.0 mL/min. Wavelength: 299, 290, 255, and 220 nm. Linearity: 5-50 µg/mL	36

Chapter-4

Experimental Work

4.1 Identification of Drugs :

Identification of drugs was carried out by melting point, UV Visible Spectroscopy and FT-IR.

Instrumentation :

- Melting Point Apparatus- T603160 (EIE Instruments Pvt. Ltd., Ahmedabad, India).
- 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.
- JASCO FT/IR-6100, (Inc. Japan) TGS Detector with Spectra Manager Software.

4.1.1 Melting Point Determination :

Melting points of GABA and NTH were determined using melting point apparatus.

The melting point of the pure drug was taken by open capillary method (Table 4.1).

Table 4.1 Melting Point of GABA and NTH

Drug	Reported Melting Point[9,11] (^{0}C)	Observed Melting Point (^{0}C)
GABA	162-166	161-164
NTH	215-220	217-221

4.1.2 UV Spectrum Determination :

Solution of NTH of 5 µg/mL was prepared in methanol and scanned in UV Visible Spectrophotometer in range of 200-400 nm to determine the absorption maxima of the drug.

Table 4.2 UV Spectrum of NTH (5 µg/mL) in Methanol

Drug	Reported Peak(nm)[11]	Observed Peak(nm)
NTH	239	239

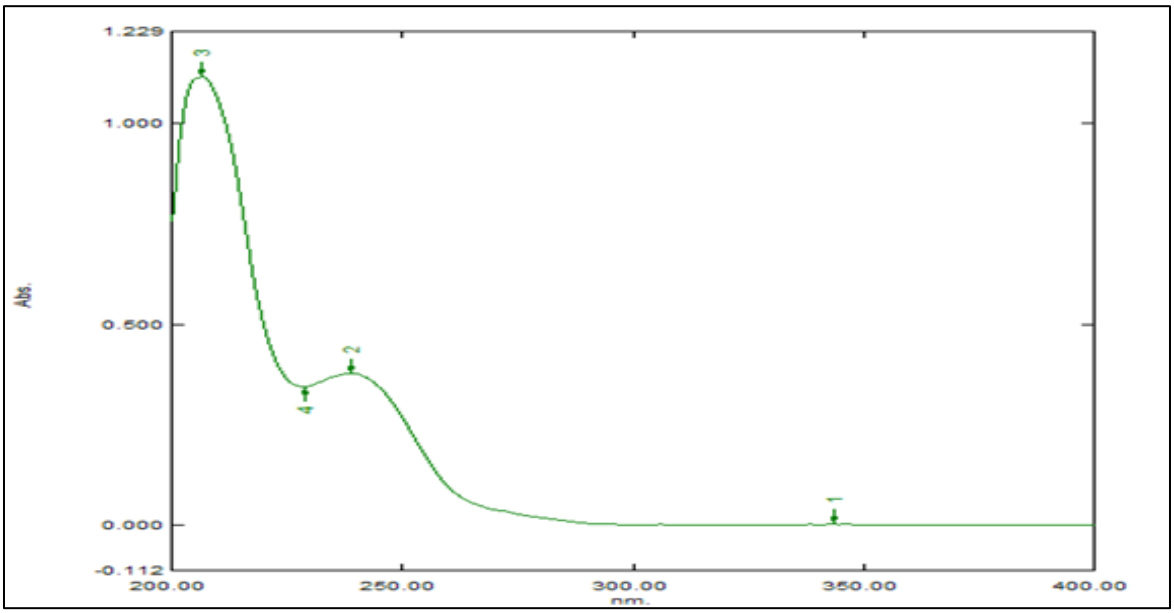


Fig.4.1: UV-spectra of NTH (5 µg/mL) in methanol

4.1.3 FT-IR Spectrum Determination

IR Spectrum of pure drugs were taken using FT-IR spectrophotometer. IR spectrum obtained were verified with the reported IR spectrum available in literature (Fig. 4.2 to 4.5). So, it can be concluded that obtained drugs are GABA and NTH in pure form.

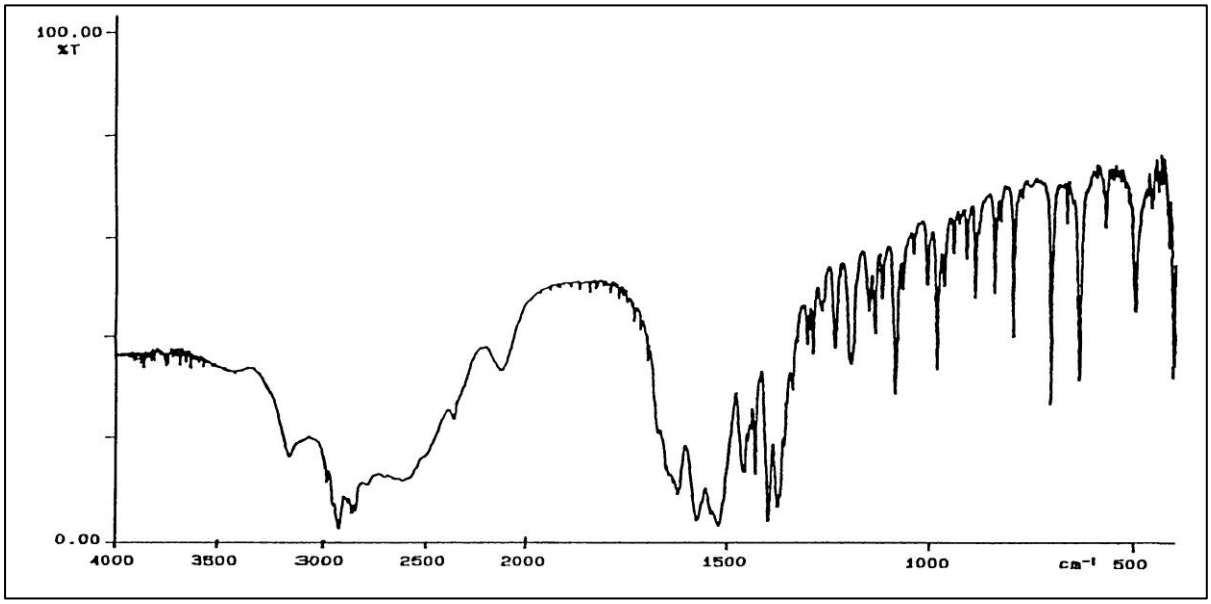


Fig. 4.2 Reported FT-IR Spectrum of GABA[37]

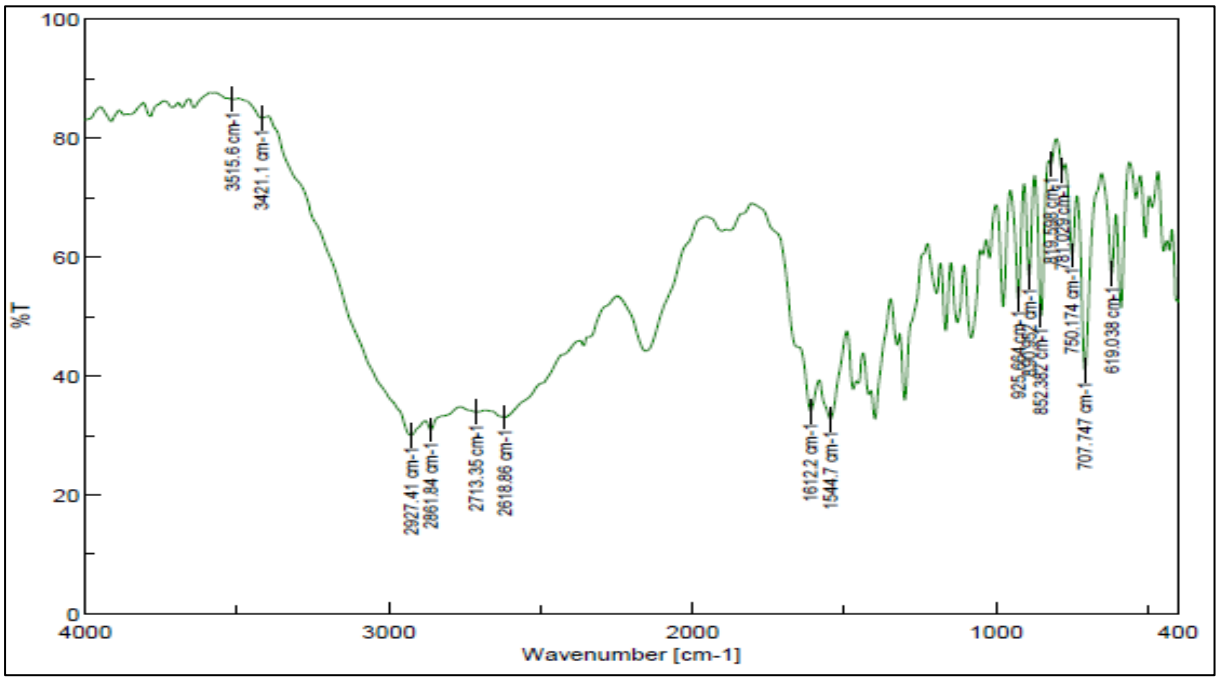


Fig. 4.3 Recorded FT- IR Spectrum of GABA

Table 4.3 Specification of FT-IR Spectra of GABA[37]

Wave No. (cm^{-1})	Functional Group
3421.1	Primary Amine
2927.41	Cyclohexane
2500-3300	Carboxylic Acid
665-910	N-H wagging

Determination of FT-IR Spectra of NTH :

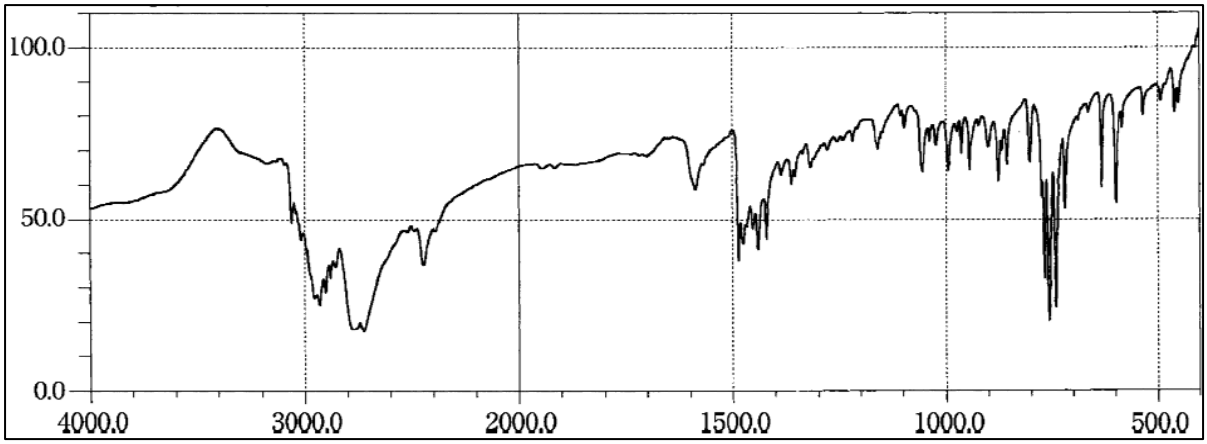


Fig. 4.4 Reported FT-IR Spectrum of NTH[38]

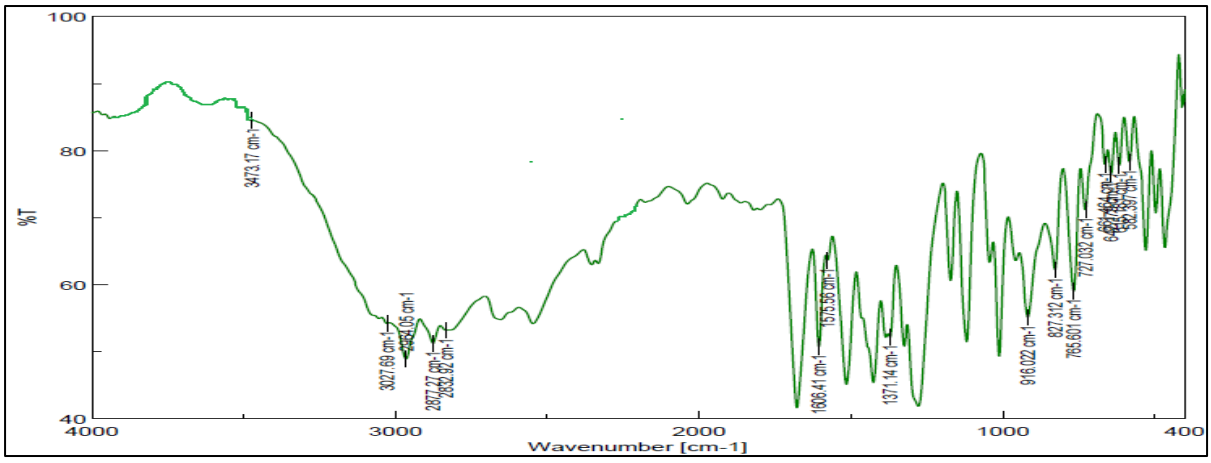


Fig. 4.5 Recorded FT-IR Spectrum of NTH

Table 4.4 Specification of FT-IR Spectra of NTH[38]

Wave No. (cm^{-1})	**Functional Group**
3473.17	Secondary Amine
2832.92-3027.69	C=C Stretch aromatic
1371.14	Aromatic Amine
582.397 - 916.022	Halides

(A) UV-Spectrophotometric Method

4.2 Simultaneous estimation of Gabapentin and Nortriptyline hydrochloride by absorbance correction UV-spectrophotometric method

4.2.1 Reagents and Materials :

- **Market Formulation (Tablet) -** Gabapin-NT (Intas Pharmaceuticals Ltd., Ahmedabad, India)
- **GABA (API) -** Gift sample from Intas Pharmaceuticals Ltd., Ahmedabad, India
- **NTH (API) -** Gift sample from Intas Pharmaceuticals Ltd., Ahmedabad, India
- **Methanol AR Grade -** S.D. Fine Chemicals Ltd., Bombay, India
- **Ortho-pthaladehyde -** AR Grade, Sisco Research Labs Pvt ltd., Bombay, India
- **β-mercaptoethanol -** (for molecular biology, AR Grade) Sisco Research Laboratories Pvt ltd., Bombay, India
- **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

4.2.2 Instrumentation :

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., Bombay, India

Sonicator Model : TRANS-O-SONIC; D-compect.,Capacity: 2 Lit., Ahmedabad, India

pH meter Model : Digital pH meter, Analab scientific Instrumentation Pvt. Ltd., Bombay, India

Water bath Model : Water bath with digital temperature controller, EIE Instrument Pvt. Ltd., Ahmedabad, India

Hot air oven Model : Hot air oven with digital temperature controller, EIE Instrument Pvt. Ltd., Ahmedabad, India

4.2.3 METHOD :

4.2.3.1 Preparation of Solution

Preparation of standard stock solution of GABA and NTH

GABA (25 mg) and NTH (25mg) were accurately weighed and transferred to two separate 25 mL amber colored volumetric flasks. Dissolved in 10 mL methanol, sonicated for 10 min and volumes were made up to mark with methanol to obtain standard stock solutions having concentration 1000 µg/mL each.

An aliquot of 5 mL from the NTH solution was transferred into 50 mL amber colored volumetric flask and volume was made up with methanol to obtain NTH working standard stock solution of 100 µg/mL.

Preparation of Sample Solution

Quantity of tablet powder (192.3375 mg) equivalent to 100 mg of GABA (and 2.5 mg of NTH) was weighed and transferred to a 100 mL amber colored volumetric flask containing about 50 mL of methanol, sonicated for 10 min. The solution was shaken and filtered through Whatmann filter paper. Then filter was washed with 5 mL methanol for two times, combined with the filtrate and volume was made up to the mark with methanol to give 1000 µg/mL GABA (25 µg/ml of NTH).

4.2.3.2 Preparation of derivatization reagent :

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 with the help of pH meter.
- After pH adjustment volume was made up 1000 mL with distilled water.

Preparation of Reagent

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

4.2.3.3 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 amber colored 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.

4.2.3.4 Optimized Experimental Conditions :

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

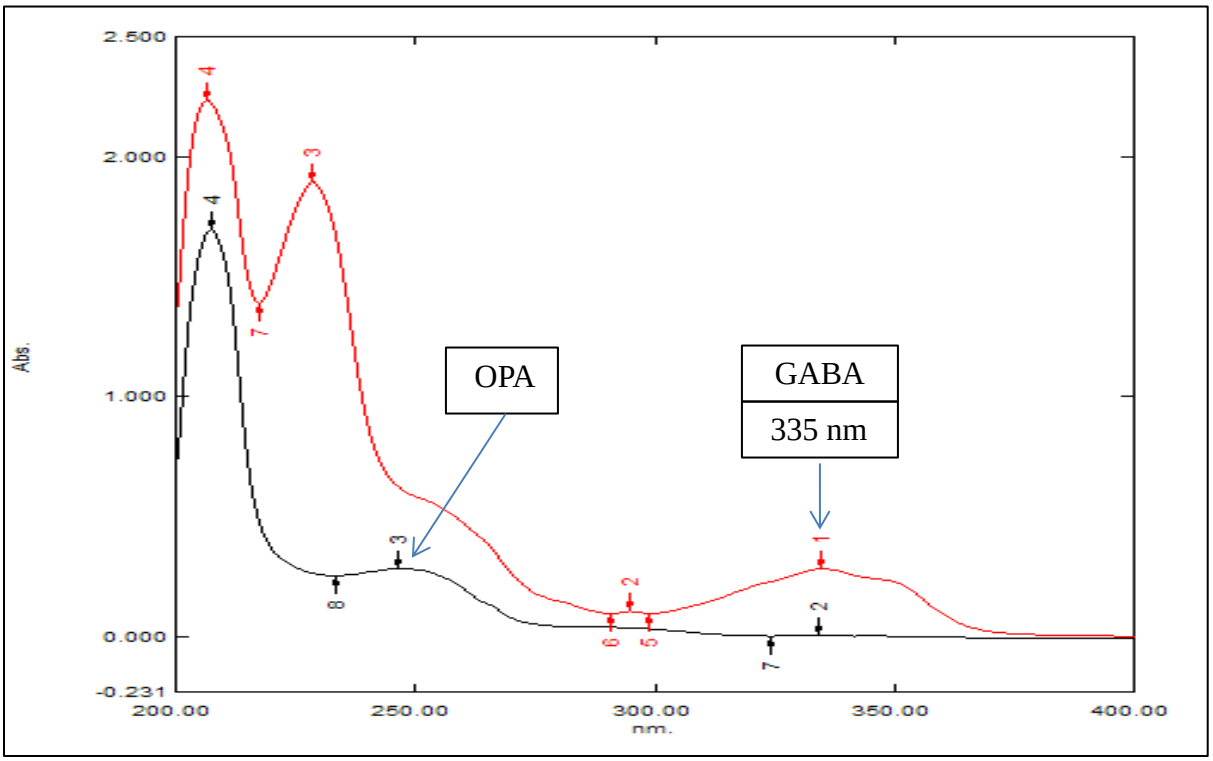


Fig. 4.6 Overlay UV-spectra of blank OPA and GABA(10 µg/mL) after Derivatization

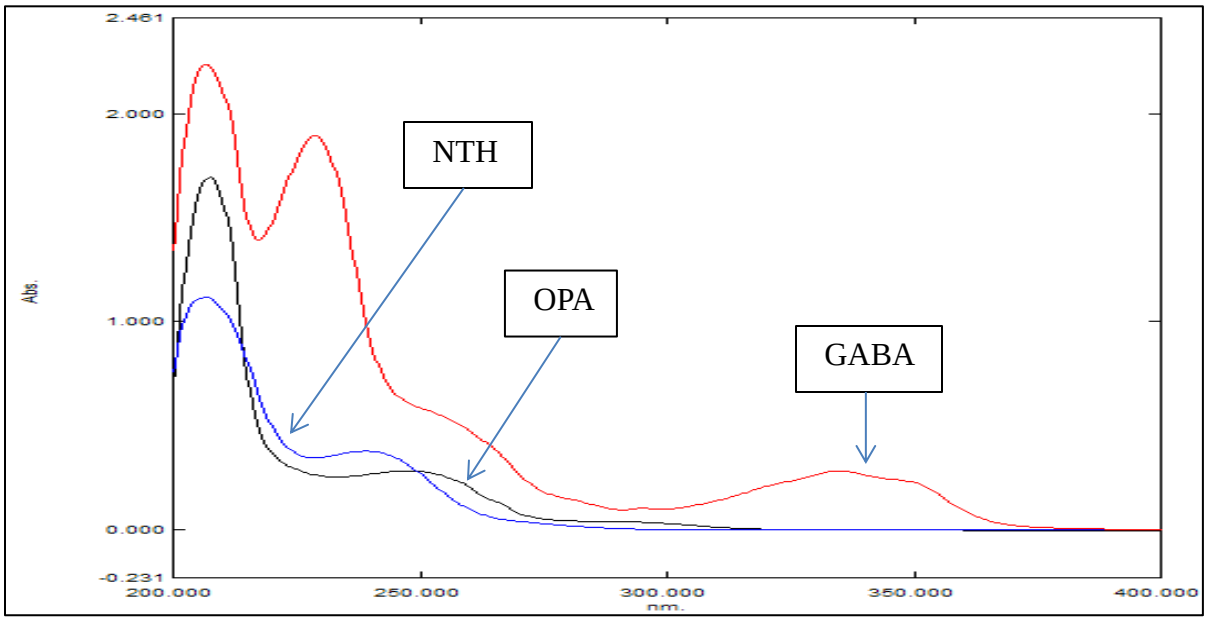


Fig. 4.7 Overlay UV-spectra of OPA(20 µg/mL), GABA(10 µg/mL) and NTH(10 µg/mL) after Derivatization

4.2.3.5 Selection of Analytical Wavelength

From the standard solutions of 100 µg/mL of both the drugs, 10 µg/mL solutions were prepared by transferring 1 mL from standard solution of both the drugs in two different 10 mL amber colored volumetric flasks respectively, derivatization reagent solution (0.1 mL) was added in volumetric flasks and were kept in water bath for 10 minutes at 70^0C. Then volume was made up with methanol and solutions 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 241 nm (λmax of NTH) and 335 nm (λmax of GABA) were selected for simultaneous analysis of both the drug using Absorbance correction method.

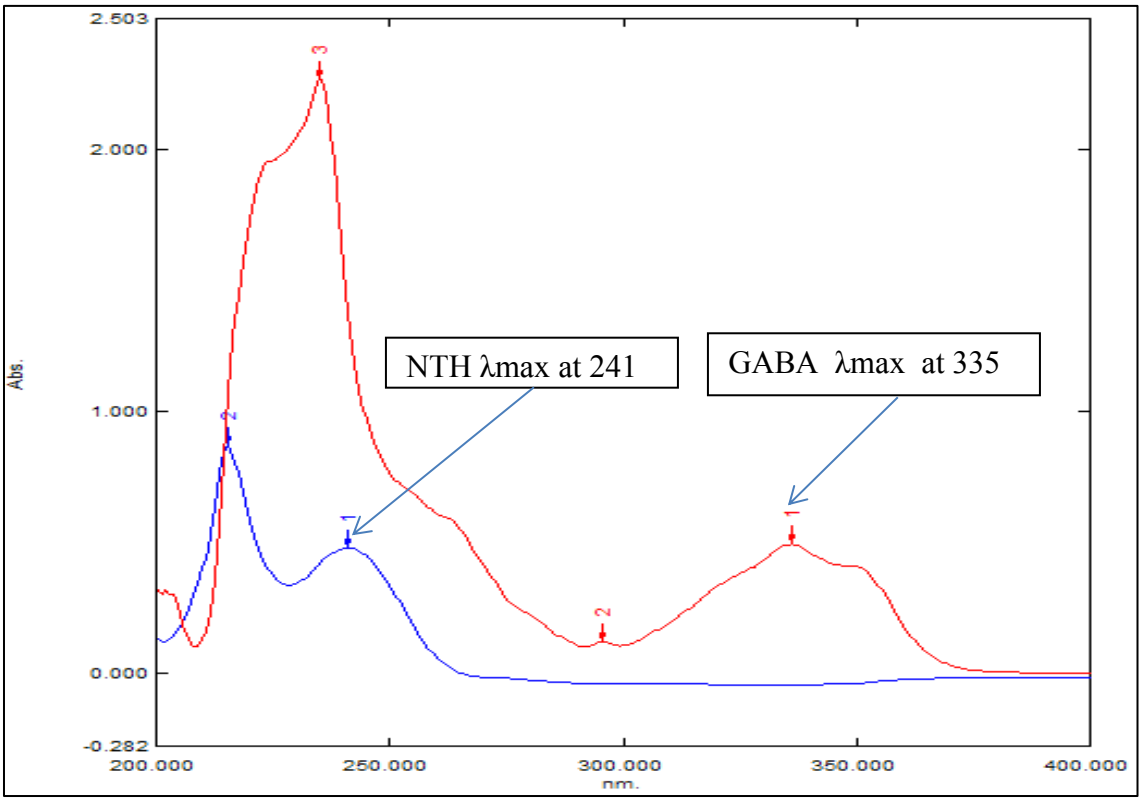


Fig. 4.8 Overlay spectra of GABA (10µg/mL) and NTH (10µg/mL) in methanol

4.2.4 VALIDATION OF UV SPECTROPHOTOMETRIC METHOD

4.2.4.1 Linearity

Preparation of calibration curve

For calibration curve of GABA, 1.6, 2.4, 3.2, 4.0, 4.8 and 5.6 mL were taken from standard stock solution(1000 µg/mL) in 10 mL amber colored volumetric flasks respectively.

For calibration curve of NTH, 0.4 , 0.6 , 0.8 , 1.0 , 1.2 and 1.4 mL were taken from standard stock solution(100 µg/mL) in 10 mL amber colored volumetric flasks respectively.

Derivatization reagent solution (0.1 mL) was added in each volumetric flask and further, same procedure was followed as per section 4.2.3.3**.** After that solutions were scanned for absorbance on 241 nm for NTH and 335 nm for GABA 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

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.

The concentrations selected for intraday and interday precision were 160, 320 and 480 µg/mL for GABA and 4, 10, 14 µg/mL for NTH. For this, 1.6, 3.2 and 4.8 mL of aliquots were pipetted out from 1000 µg/ml standard solution of GABA and 1.6, 4 and 5.6 mL of aliquot was pipetted out from 25 µg/mL standard solution of NTH, transferred to individual nine 10 mL amber colored volumetric flasks and 0.1 mL of derivatization reagent solution was added in each volumetric flask and further same procedure was followed as per section 4.2.3.3**.**

After that absorbance of each solution was measured three times in a day for intraday precision and at three different days for interday precision at their estimating wavelengths .

4.2.4.2.2 Repeatability

To study the repeatability, six determinations of particular concentration (i.e. 320 µg/mL for GABA and 10 µg/mL for NTH) were carried out. For repeatability study, 3.2 mL and 4 mL were pipetted out from standard stock solutions of GABA (1000 µg/mL) and NTH (25 µg/mL) respectively and transferred into separate 10 mL amber colored volumetric flasks and 0.1 mL of derivatization reagent solution was added in each volumetric flask and further same procedure was followed as per section 4.2.3.3**.** The volume was made upto 10 mL with methanol to obtain solutions of 320 µg/mL and 10 µg/mL concentrations of GABA and NTH respectively. Each solution was scanned six times at their estimating wavelengths.

4.2.4.3 Accuracy

The accuracy of the method was determined by performing the recovery studies from previously analyzed tablet sample by standard addition method at three different levels (80,100,120 %).

For GABA accuracy studies, 2.4 mL was taken from 1000 µg/mL sample solution (1000 µg/mL GABA) in three different 10 mL amber colored volumetric flasks and 1.92 mL, 2.4 mL, 2.88 mL (80%, 100% and 120% of test sample concentration) of working standard GABA solution was added and followed by 0.1 mL of derivatization reagent solution was added in the volumetric flask and further same procedure was followed as per section 4.2.3.3. After that solution were measured for absorbance at 335 nm.

For NTH accuracy studies, 2.4 mL was taken from 1000 µg/mL sample solution (25 µg/mL NTH) in three different 10 mL amber colored volumetric flask and 1.92 mL, 2.4 mL, 2.88 mL (80%, 100% and 120% of test sample concentration) was taken from working standard solution in 10 mL amber colored volumetric flask and 0.1 mL of derivatization reagent solution was added in volumetric flask and further same procedure was followed as per section 4.2.3.3. After that solution were scanned for absorbance on 241 nm.

4.2.4.4 Limit of detection and Limit of quantification (LOD and LOQ)

For LOD and LOQ determination, Calibration curve for both the drugs was repeated six times. They were measured by using mathematical equations given below :

LOD = 3.3 x σ / S

LOQ = 10 x σ / S

Where, σ = standard deviation of intercept

s = slope of straight line

4.2.4.5 Robustness

Robustness was performed on concentrations (320 µg/mL) of GABA and (10 µg/mL) of NTH. The solutions were prepared as per section 4.2.3.3. Robustness of the method was determined by making change in λmax 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.

4.2.4.6 Analysis of GABA and NTH in combination tablet dosage form

Prepared the sample solution (as per section 4.2.3.1), 0.1 mL solution was taken in 10 mL amber colored volumetric flask and further same procedure was followed as per section 4.2.3.3**.** After that solution was scanned for absorbance at 241 nm and 335 nm.

(B) Spectrofluorimetric Method

4.3 Simultaneous estimation of gabapentin and nortriptyline hydrochloride by synchronous Spectrofluorimetric method

4.3.1 Reagents and Materials :

- **Market Formulation (Tablet) -** Gabapin-NT (Intas Pharmaceuticals Ltd., Ahmedabad, India)
- **GABA (API) -** Gift sample from Intas Pharmaceuticals Ltd., Ahmedabad, India
- **NTH (API) -** Gift sample from Intas Pharmaceuticals Ltd., Ahmedabad, India
- **Methanol AR Grade -** S.D. Fine Chemicals Ltd., Bombay, India
- **Ortho-pthaladehyde -** AR Grade, Sisco Research Labs Pvt ltd., Bombay, India
- **β-mercaptoethanol -** (for molecular biology, AR Grade), Sisco Research Laboratories Pvt ltd., Bombay, India
- **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
- **Double distilled water**

4.3.2 Instrumentation :

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, Bombay, India

Sonicator Model : TRANS-O-SONIC; D-compect., Capacity: 2 Lit., Ahmedabad, India

pH meter Model : Digital pH meter, Analab scientific Instrumentation Pvt. Ltd., Bombay, India

Water bath Model : Water bath with digital temperature controller, EIE Instrument Pvt. Ltd., Ahmedabad, India

Hot air oven Model : Hot air oven with digital temperature controller, EIE Instrument Pvt. Ltd., Ahmedabad, India

4.3.3 METHOD :

4.3.3.1 Preparation of Solution

Preparation of standard stock solution of GABA and NTH

GABA (25 mg) and NTH (25mg) were accurately weighed and transferred to two separate 25 mL amber colored volumetric flasks. Dissolved in 10 mL double distilled water, sonicated for 10 min and volumes were made up to mark with double distilled water to obtain standard stock solutions having concentration 1000 µg/mL each.

An aliquot of 5 mL from the GABA and NTH solution was transferred into 50 mL amber colored volumetric flask and volume was made up with double distilled water to obtain standard stock solutions of 100 µg/mL of both drugs. From the standard solution of NTH an aliquot of 6.25 mL was transferred into 25 mL amber colored volumetric flask and volume was made up with double distilled water to obtain 25 µg/mL working standard solution of NTH.

Preparation of Sample Solution

Quantity of tablet powder (192.3375 mg) equivalent to 100 mg of GABA (and 2.5 mg of NTH) was weighed and transferred to a 100 mL amber colored volumetric flask containing about 50 mL of double distilled water, sonicated for 10 min. The solution was shaken and filtered through whatmann filter paper. Then filter was washed with double distilled water, combined with the filtrate and volume was made up to the mark with double distilled water to give 1000 µg/mL GABA (25 µg/ml of NTH). After that an aliquot of 5 mL was transferred to 50 mL amber colored volumetric flask and volume was made up with double distilled water to obtain 100 µg/mL GABA sample solution.

4.3.3.2 Preparation of derivatization reagent :

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 with the help of pH meter.
- After pH adjustment volume was made up 1000 mL with distilled water.

Preparation of Reagent

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

4.3.3.3 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 solution was taken in 10mL amber colored volumetric flask. 0.1 mL of derivatization reagent solution was added. The solution was kept on water bath for 10 minutes at 70^0C. Then volume was made up with borate buffer 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.

4.3.3.4 OPTIMIZED EXPERIMENTAL CONDITIONS

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

4.3.3.5 SELECTION OF ANALYTICAL WAVELENGTH

From the working standard solutions, 10 µg/mL solutions of both the drugs were prepared by transferring 1 mL from 100 µg/mL standard solution of GABA and 4 mL from 25 µg/mL standard solution of NTH in 10 mL amber colored volumetric flasks of both the drugs respectively. Derivatization reagent solution (0.1 mL) was added in volumetric flask and further same procedure was followed as per section 4.3.3.3. After that solution were 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 417 nm (λ_{emi} of GABA) selected for estimation of GABA and 377 nm (λ_{emi} of NTH) for estimation of NTH were selected for analysis.

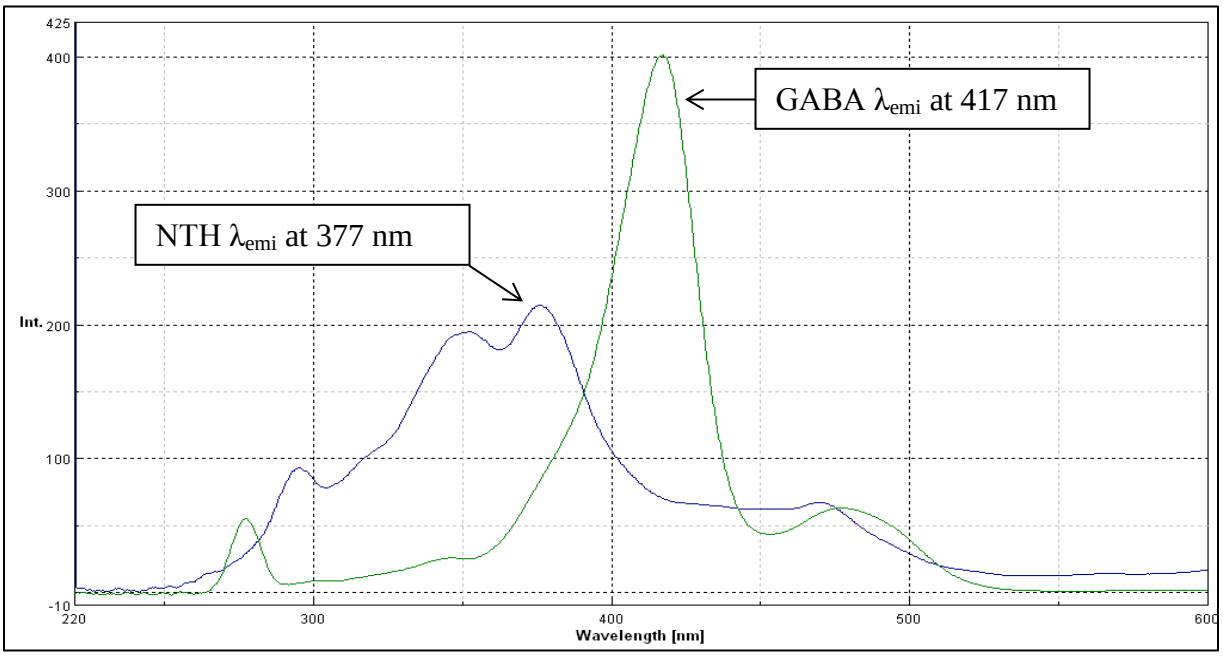


Fig. 4.9 Overlay synchronous spctrofluorimetric spectra of GABA and NTH in borate buffer

4.3.4 VALIDATION OF SPECTROFLUORIMETRIC METHOD

4.3.4.1 Linearity

Preparation of calibration curve

For calibration curve of GABA, 1, 2, 3, 4, 5, 6, 7, 8 and 9 mL were taken from working standard stock solution (100 µg/mL) of GABA in 10 mL amber colored volumetric flasks respectively.

For calibration curve of NTH, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 0.9 mL were taken from the standard stock solution (25 µg/mL) of NTH in 10 mL amber colored volumetric flasks respectively.

Derivatization reagent solution (0.1 mL) was added in each volumetric flask and further, same procedure was followed as per section 4.3.3.3**.** After that solutions were scanned for intensity on 417 nm for GABA and 377 nm for NTH and scanning was done.

4.3.4.2 Precision

4.3.4.2.1 Intra-day and Interday precision

Intra-day and Inter-day precision was determined by measuring the intensity of both the drugs three times within a day and on three different days, respectively.

The concentrations selected for intraday and interday precision were 20, 50 and 80 µg/mL for GABA and 0.5, 1.25, 2 µg/mL for NTH. For this, 2, 5 and 8 mL of aliquots were pipetted out from 100 µg/ml standard solution of GABA and 0.2, 0.5 and 0.8 mL of aliquot was pipetted out from 25 µg/mL standard solution of NTH, transferred to individual nine 10 mL amber colored volumetric flasks and 0.1 mL of derivatization reagent solution was added in each volumetric flask and further same procedure was followed as per section 4.3.3.3**.**

After that intensity of each solution was measured three times in a day for intraday precision and at three different days for interday precision at their estimating wavelengths.

4.3.4.2.2 Repeatability

To study the repeatability, six determinations of particular concentration (i.e. 50 µg/mL for GABA and 1.25 µg/mL for NTH) were carried out. For repeatability study, 5 mL and 0.5 mL were pipetted out from standard stock solutions of GABA (100 µg/mL) and NTH (25 µg/mL) respectively and transferred into separate 10 mL amber colored volumetric flasks and 0.1 mL of derivatization reagent solution was added in each volumetric flask and further same procedure was followed as per section 4.3.3.3**.** The volume was made upto 10 mL with methanol to obtain solutions of 50 µg/mL and 1.25 µg/mL concentrations of GABA and NTH respectively. Each solution was scanned six times at their estimating wavelengths.

4.3.4.3 Accuracy

The accuracy of the method was determined by performing the recovery studies from previously analyzed tablet sample by standard addition method at three different levels (80,100,120 %).

For GABA accuracy studies, 3.0 mL was taken from 1000 µg/mL sample solution (100 µg/mL GABA) in three different 10 mL amber colored volumetric flasks and 2.4 mL, 3.0 mL, 3.6 mL (80%, 100% and 120% of test sample concentration) of working standard GABA 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 4.3.3.3. After that solution were measured for intensity at 417 nm.

For NTH accuracy studies, 0.4 mL was taken from 1000 µg/mL sample solution (25 µg/mL NTH) in three different 10 mL amber colored volumetric flasks and 0.32 mL, 0.4 mL, 0.48 mL (80%, 100% and 120% of test sample concentration) was taken from working standard solution in 10 mL amber colored volumetric flask and 0.1 mL of derivatization reagent solution was added in volumetric flask and further same procedure was followed as per section 4.3.3.3. After that solutions were scanned for intensity on 377 nm.

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

For LOD and LOQ determination, Calibration curve for both the drugs was repeated six times. They were measured by using mathematical equations given below :

LOD = 3.3 x σ / S

LOQ = 10 x σ / S

Where, σ = standard deviation of intercept

s = slope of straight line

4.3.4.5 Robustness

Robustness was performed on concentrations (50 µg/mL) of GABA and (1.25 µg/mL) of NTH. The solutions were prepared as per section 4.3.3.3. Robustness of the method was determined by making change in λmax 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.

4.3.4.6 Analysis of GABA and NTH in combination tablet dosage form

Prepared the sample solution (as per section 4.3.3.1), 0.1 mL solution was taken in 10 mL amber colored volumetric flask and further same procedure was followed as per section 4.3.3.3**.** After that solution was scanned for intensity at 377 nm and 417 nm.

Chapter-6
Comparison and Conclusion

Comparison

Comparison of developed UV-spectrophotometric and spectrofluorimetric methods was performed by applying unpaired t- test . The results are shown in table 6.1 and 6.2.

Table 6.1 Assay Results of the Proposed Methods

Drug	% Assay	
	UV Method	**Spectrofluori method**
GABA	99.36	99.56
	99.51	99.10
	99.29	99.68
NTH	98.30	98.21
	98.45	99.02
	98.11	98.76

Table 6.2 Results of unpaired t-test

Parameter	GABA		NTH	
	UV method	**Spectrofluori method**	**UV method**	**Spectrofluori method**
Mean % Asaay	99.39	99.35	98.29	98.67
Variance	1.595	0.847	1.349	0.432
Observations	3	3	3	3
Degree of Freedom	2	2	2	2
P Value	0.1204		0.385	

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 GABA and NTH in combined tablet dosage form. The excipients usually present in the pharmaceutical formulation did not interfere with determination of GABA and NTH. Any of the developed method can be successfully used for routine quality control of the GABA and NTH 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 less sensitivity as compare to UV spectrophotometric method And 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.

Chapter-7
Summary

UV spectrophotometric, spectrofluorimetric methods were developed and validated for the simultaneous estimation of GABA and NTH in bulk and pharmaceutical formulation. Spectrofluorimetric method was applied for estimation of GABA and NTH. OPA-β-mercaptoethanol derivatization of GABA was applied in both UV spectrophotometric and spectrofluorimetric methods because of absence of chromophoric group in GABA.

Derivatization process includes reaction of GABA with OPA in presence of β-mercaptoethanol under alkaline medium results in OPA-GABA derivative having λmax 335 nm in methanol for UV method and λexci 275 nm, 417 nm λemi 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 absorbance correction method that involves measurement of absorbances at two wavelengths i.e. at 335 nm (λmax of GABA) and 241 nm (λmax of NTH) in methanol. Linearity range was observed in the concentration range 160-560 µg/mL with mean recovery of 100.96 % ± 0.6769 for GABA and 4-14 µg/mL with mean recovery of 99.73 % ± 0.5502 for NTH respectively. The correlation coefficients for GABA and NTH were found to be 0.9994 and 0.9999, respectively. LOD and LOQ were found to be 2.390 and 7.241 µg/mL for GABA 0.010 and 0.030 µg/mL for NTH, respectively. The R.S.D. values for precision studies were found to be less than 2 for both the drugs.

Spectrofluorimetric method includes synchronous simultaneous equation method using delta value 65 (medium sensitivity mode) that involves measurement of fluorescence intensity at two wavelengths in synchronous spectra of drugs i.e. at 417 nm for GABA (λ_{emi} of GABA) and 377 nm (λ_{emi} of NTH) in borate buffer. Linearity range was observed in the concentration range 10-90 µg/mL with mean recovery of 100.73 % ± 0.7422 for GABA and 0.25-2.25

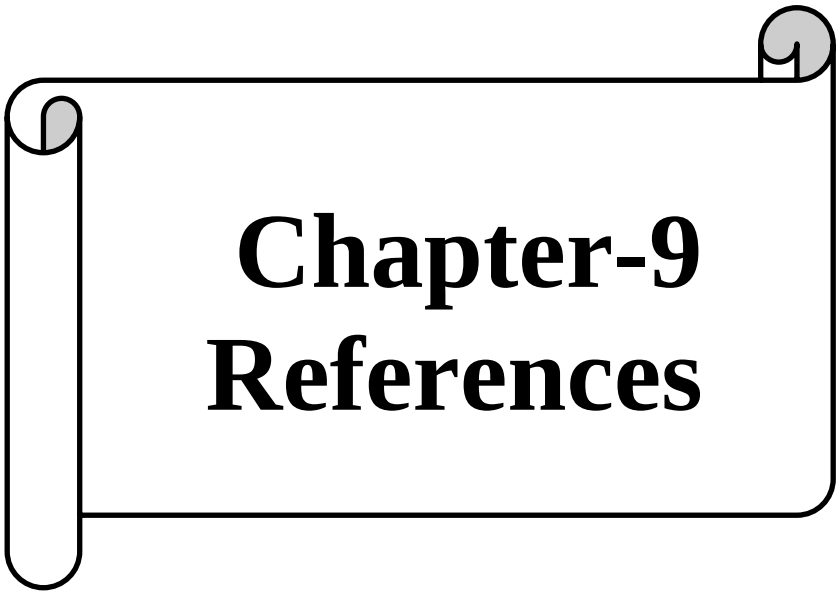
μg/mL with mean recovery of 100.08 % ± 0.1744 for NTH, respectively. The correlation coefficients for GABA and NTH were found to be 0.9989 and 0.9984, respectively.
LOD and LOQ were found to be 0.596 and 1.806 μg/mL for GABA and 0.060 and 0.181 μg/mL for NTH, respectively. The RSD values for precision 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.

Chapter-8
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 HPTLC, HPLC and Super critical fluid chromatography (SFC) can be developed for this combination, Stability indicating different techniques can be developed for the same.



Chapter-9
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