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ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR SELECTED ANTI INSOMNIA DRUG IN BULK AND FORMULATED PRODUCT

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ABSTRACT

A simple precise, rapid and accurate UV-Visible Spectrophotometer and High Performance Liquid Chromatography Method was developed for simultaneous estimation of Melatonin and L- Theanine, in pharmaceutical dosage form. This method was carried out on Inertsil-ODS C18 (250x4.6 mm, 5µ) column with a mobile phase consisting of Methanol: Buffer (60:40v/v). me of The retention time of Melatonin and L-Theanine is 4.25min and 5.99 min respectively. The flow rate was 1.0 ml/min with UV detection at 278 nm. The linear regression data for the linearity plot showed good relationship with correlation coefficient value for Melatonin and L-Theanine were r^2= 0.999 and r^2= 0.999. The stock solution equivalent to20ppm to 80ppm with respect to both drugs are prepared in combination of Melatonin and L-Theanine. The relative standard deviation for intra-day precision has been found to lower than 2.0%.0 The method is validated According to ICH guidelines. The development was validated in terms of specificity, estimated by accuracy, linearity, limit of detection, limit of quantification and solution stability. Sensitivity was estimated by determination of LOD and LOQ and values are found to be 0.25 and 0.77 for Melatonin and 0.34 and 1.05 for L-Theanine. The purposed method can be successfully applied for the determination of these drugs in combined dosage forms.

KEYWORDS: melatonin, L-Theanine, UV Visible Spectrophotometer, High Performance Liquid Chromatography, Method Validation, ICH guidelines.

INTRODUCTION

Pharmaceutical analysis is a crucial discipline that ensures the quality control and assurance of bulk drugs and their formulations. Subfield of analytical chemistry that is concerned with identification, quantification, and separation of constituents in a particular sample.

Maintaining the safety, effectiveness, and consistency of pharmaceutical products is largely dependent on this field, which includes both qualitative and quantitative chemical characterisation1 In recent years, several analytical techniques have been created.

1.0 INTRODUCTION TO SIMUTANEOUS ESTIMATION METHOD

1.0.1 SPECTROPHOTOMEETRIC APPROACHES

Because it is less costly to buy and requires less upkeep, spectrophotometry is frequently used, particularly by small firms. Analysis method quantifies how much monochromatic light in the 200–380 nm band of the near ultraviolet spectrum is absorbed by colorless materials. Photometric analysis is based on Bouger- Lambert-Beer's law, which states that a solution's absorbance is directly proportional to the analyte's concentration. The basic idea behind an ultraviolet spectrophotometer is that light with a specific wavelength interval travels through a solvent-filled cell and lands on a photoelectric cell, which converts the radiant energy into electrical energy that can be measured by a galvanometer. 2

1.0.1.1 The important applications are

1.0.1.1.1 Numerous kinds of organic, inorganic, and ionic molecules can be identified.

1.0.1.1.2 Numerous biological, organic, and inorganic species can be determined quantitatively.

Chromatographic effluent identification & monitoring. Finding two or more compounds in a single sample using spectrophotometry without first separating them is one of the biggest problems analytical chemists face. Pharmaceutical compounds are frequently analyzed using the ultra-violet (UV) spectroscopic approach, most likely due to its great sensitivity and affordability. This method estimates the drug content in formulations using both direct and indirect measuring techniques.

However, over the past three decades, many analytical research scientists have been captivated by indirect measurement techniques like derivative spectrophotometry and ratio spectra derivative spectrometric approaches. This approach has led to the development of

numerous potential techniques for the estimate of multicomponent in pharmaceutical formulations.

1.0.1.2 Simultaneous Equation Method

Given particular conditions, it could be possible to recognize mutually medicines utilizing the concurrent equation methodology if a sample contains 2 absorbing medications, each of which absorbs at other's λ max. Data needed is 1.0.1.2.1 At λ1 and λ2, x's absorptivity is ax1 and ax2, respectively. 1.0.1.2.2 At λ1 and λ2, y's absorptivity is ay1 and ay2, respectively.

1.0.1.2.3 The absorbance of the diluted samples at λ1 and λ2, A1 and A2 respectively.

Let Cx and Cy be the concentrations of x and y respectively in the diluted samples. Two equations are constructed base upon the fact that at λ1, the absorbance of the mixture is the sum of the individual absorbance of x and y.

A1 = ax1 b cx + ay1 b cy (1)

A2 = ax2 b cx + ay2 b cy (2)

For measurements in 1 cm cells, b=1cm. rearrange Eq. (2) Cy = (A2 – ax2 cx)/ay2 (3)

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

Cx = (A2ay1 – A1ay2) / (ax2ay1 – ax1 ay2) (4)

Cy = (A1ax2 – A2ax1) / (ax2ay1 – ax1ay2)… (5)

Proposed absorbance ratios that place restrictions on the relative concentrations of the mixture's component components serve as the basis for the requirements for reaching the highest degree of precision. The ratios (A2/A1) / (ax2/ax1) and (ay2/ay1) / (A2/A1) must be outside of the range 0.1-2.0 in order to precisely determine y and x, respectively. These criteria only hold when the λ maxes of the two components are sufficiently diverse and there is no chemical interaction between them, hence disproving the earlier premise that the overall absorbance is the sum of the separate absorbances. The simultaneous equation approach was developed to determine various mixtures simultaneously. 3-6

1.0.2 TECHNIQUE VALIDATION PARAMETERS

The process by which laboratory tests demonstrate that an analytical method's performance characteristics satisfy the demands of the intended analytical application is known as validation. Any new or altered procedure needs to be verified to ensure that it can yield reliable and reproducible results when used by numerous operators in the same or various

labs using the same equipment. The specific approach and its intended applications determine the kind of validation procedure that is needed. The quality, consistency, and dependability of analytical results can be assessed using the results of method validation, which is an essential component of any sound analytical process. Using equipment that is within specification, functioning as intended, and calibrated correctly is a crucial part of the method validation process. 7

1.0.2.1 HPLC METHOD DEVELOPMENT

The processes that are used to separate the various species in a mixture according to how they distribute between a stationary and a mobile phase are called "chromatography."

1.0.3 METHODS OF CHROMATOGRAPHY

Modes of chromatography are defined essentially according to the nature of the interactions between the solute and the stationary phase, which may arise from hydrogen bonding, Vander walls forces, electrostatic forces or hydrophobic forces or basing on the size of the particles (e.g. Size exclusion chromatography).

1.0.3.1 The following are the various chromatography modes

1.0.3.1.1 Normal Phase Chromatography

1.0.3.1.2 Reversed Phase Chromatography

1.0.3.1.3 Reversed Phase – ion pair Chromatography

1.0.3.1.4 Ion-Exchange Chromatography

1.0.3.1.5 Size Exclusion Chromatography

1.0.2.1.1 ADSORPTION CHROMATOGRAPHY OR NORMAL PHASE CHROMATOGRAPHY

A polar adsorbent serves as the stationary phase in normal phase chromatography, whereas a combination of non-aqueous solvents typically serves as the mobile phase. The silanol groups are saturated near the end of the silica structure. The entire surface of these OH groupings is statistically disorganized. The highly polar active sites are reflected by the silanol groups in the stationary phase. These circumstances occur when a molecule has one or more atoms with a double bond or a single pair of electrons. The following is the sequence in which the adsorption strengths and, consequently, the k' values (elution series), rise. Olefins, aromatics, organic halogen compounds, sulphides, ethers, esters, aldehydes, ketones, amines, sulphones, amides, and carboxylic acids are all classified below saturated hydrocarbons. In addition to

the functional groups found in the sample molecule, steric variables also affect the intensity of interactions. In a molecule with many functional groups, the more polar functional group dictates the reaction's characteristics. Additional separation optimization possibilities and chances for particular interactions between the analyte and the stationary phases are offered by the aminopropyl and cyanopropyl phases.

1.0.3.1.2 REVERSED PHASE CHROMATOGRAPHY

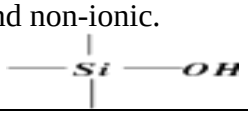
Making less polar or non-polar was the goal in order to separate water-soluble polar chemicals using polar solvents. As a result, the ionic character of the chemically modified silica has been inverted, resulting in a reversed phase or non-polarity. Such silica is used in a chromatographic separation technique known as reversed-phase chromatography.

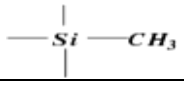
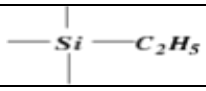
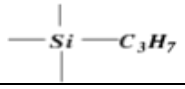
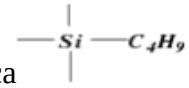
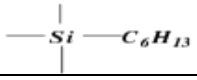
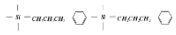
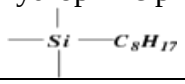
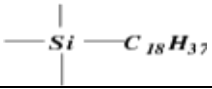
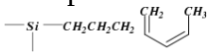
Table no. 1: Classification of Chromatographic Methods.

Stationary phase	Mobile phase	Method
Solid	Liquid	Adsorption column, thin-layer, ion exchange, High performance liquid chromatography.
Liquid	Liquid	Partition, column, thin-layer, HPLC, paper chromatography.
	Gas	Gas–Liquid Chromatography

High performance, high resolution, high speed, and high pressure define HPLC, a sophisticated type of column chromatography. In HPLC, the mobile phase is rapidly forced through the column, drastically reducing analysis time compared to traditional methods and allowing the use of smaller support particles, which enhances column efficiency. An eluent reservoir, high pressure pump, sample injector, stationary phase column, detector, and recorder are the fundamental parts of an HPLC system. The development of highly efficient microparticulate bound phases has broadened HPLC's applications and improved its capability to analyze complex mixtures.

B. BONDED PHASES FOR HPLC AND THEIR ABBREVIATIONS

	Phase	Description
	Si	**Silica** traditional normal phase substance. ideal for separating organic molecules that are polar and non-ionic. 
	C1	**TMS, SAS**, Tri methyl silane substance in reverse phase. exceptional selectivity for multifunctional

		and polar substances. For non-polar liquids, the least retentive phase of all alkyl group bonds 
C2		**RP-2, Dimethyl** lesser retentive than C4, C8, or C18, reversed phase material. Retention is higher than C1.
		
C3	**Propyl:** Hydrophobic interaction chromatography (HIC) of proteins and peptides uses reversed phase material. 	
C4	**Butyl**: For non-polar solutes, reversed phase material, which is helpful in ion-pairing chromatography, provides less retention than C8 and C18 phases. It is the perfect phase for examining big proteins and hydrophobic peptides when it is bound to 300 Å  silica	
C6	**Hexyl**: Ion-pairing chromatography can benefit from this reversed phase material. less retentive than stages C8 and C18. 	
C8	**MOS, RP-8, LC8, Octyl**  substance in reverse phase; less retentive than C18, but with comparable selectivity. broad applicability (e.g., steroids, nucleosides, medicines). It is the perfect phase for peptides, peptide mapping, and tiny hydrophilic proteins when bound to 300 Å silica. 	
C18	**ODS, RP-18, LC18, Octadecyl** Conventional reversed phase material is especially retentive for non-polar solutes and performs well in ion-pairing chromatography. The assay of nucleosides, nucleotides, steroids, medications, vitamins, fatty acids, and environmental chemicals can all benefit from this phase, which is best suited for separating small hydrophilic peptides when combined with 300 Å silica 	
C6H5	It has special selectivity and is a reversed phase material. For the analysis of aromatic chemicals, it is helpful. This phase is helpful for HIC when bound to 300 Å silica.  Phenyl	

C. Derivatization

When direct detection is insufficient in HPLC, derivatization is used to increase detection sensitivity and selectivity. Creating UV-absorbing or luminous derivatives is a common derivatization technique. N-succinimidyl p-nitrophenyl acetate, phenyl hydrazine, and 3, 5-

dinitrobenzyl chloride are examples of UV reagents, whereas dansyl chloride, 4-bromomethyl-7-methoxycoumarin, and fluorescamine can be used to create fluorescent derivatives. Derivatization can be carried out by online reactions between the detector and column outlet or prior to sample injection.

D. Gradient elution

In order to progressively increase solvent strength, gradient elution, also known as solvent programming, entails altering the solvent composition during separation. This technique is ideal for analyzing complex or unknown samples, as it provides good resolution across a wide polarity range. Two gradient systems exist: low-pressure systems mix solvents at atmospheric pressure before pumping to the column, while high-pressure systems pump solvents into a mixing chamber under high pressure before entering the column.

E. Performance calculations

To access overall system performance, the following values are calculated and may be included in a custom report.

Relative retention, Theoretical plates, Capacity factor Resolution, Peak asymmetry, Plates per meter

These system performance figures for the separation of two chromatographic components were computed using the following settings.

Relative retention (Selectivity)

a = **(t**2 - **ta) / (t**1 - **ta).**

Theoretical plates

n = 16 (t / W) 2

1

Capacity factor

K' = (t2 / ta) $^{-1}$

Resolution

R = 2 (t2 - t1) / (W2 + W1)

Peak asymmetry

T = W0.05 / 2f

Plates per meter

N = n / L

HETP: L/n

Where, a = Relative retention.

t2 = Retention time of the second peak measured from point of injection. t1 = Retention time of the first peak measured from point of injection.

ta = Retention time of an inert peak not retained by the column, measured from point of injection.

n = Theoretical plates.

t = Retention time of the component.

W = Width of the base of the component peak using tangent method. K' = Capacity factor.

R = Resolution between a peak of interest (p2) and the peak preceding it (p1)

W2 = Width of the base of component peak 2. W1 = Width of the base of component peak 1.

T = Peak asymmetry, or tailing factor.

W0.05 = Distance from the leading edge to the tailing edge of the peak, Measured at a point 5 % of the peak height from the baseline.

f = Distance from the peak maximum to the leading edge of the peak. L = length of column, in meters

N = Number of plates per meter

E. METHOD OPTIMISATION

Column/stationary phase selection: The first and most crucial stage in developing a method is choosing the column. When choosing chromatographic columns, some crucial factors to take into account are:

i. Length and diameter of the column.
ii. Packing material.
iii. Shape of the Length particles.
iv. Size of the particles.

1.3 TECHNIQUE VALIDATION

The process of creating recorded proof that an analytical method regularly yields results that satisfy predetermined criteria and quality requirements is known as method validation, according to ICH. Verifying that the process is appropriate for its intended application in determining the identification, quality, purity, and potency of drug substances and products is

an essential step in the method development process. Method validation essentially demonstrates an analytical method's correctness and dependability for its particular use. Key analytical parameters are more consistently validated for chromatographic procedures used in analytical applications.

For chromatographic methods used in analytical applications there is more consistency in validation practice with key analytical parameters

(a) Recovery (b) Response function (c) Sensitivity (d) Presicion (e) Accuracy (f) limits of detection (g) Limit of quantitation (h) Ruggedness (i) Robustness (j) stability (k) system suitability

a) Recovery

The response of a processed spiked matrix standard expressed as a percentage of the response of a pure standard that has not undergone sample pretreatment is the analytical method's absolute recovery. This indicates if the process is able to detect the whole amount of analyte in the sample.

$$\text{Absolute recovery} = \frac{\text{Response of an analyte spike into matrix (processed)}}{\text{Response of analyte of pure standard (unprocessed)}} \times 100$$

b) Sensitivity

When slight variations in concentration cause notable shifts in reaction, the analytical technique is said to be sensitive. The calibration curve's slope indicates sensitivity. The dynamic range, or quantification limits, of the method specify the highest and lowest concentrations that may be measured with a reasonable degree of precision; these limits are usually within ±15%. Overrange samples need to be diluted in a drug-free matrix and reanalyzed; concentrations outside of this range shouldn't be inferred from the calibration curve.

c) Precision

The main goal of an analytical determination is to obtain a valid estimate of the true value. When selecting an analytical method, precision and accuracy are key considerations, as they determine the overall error of a measurement. Precision refers to the reproducibility of results within a set of replicate measurements and indicates the degree of scatter around a central

value. A common statistical measure of precision is the standard deviation, which quantifies the spread of results in a dataset. It is calculated using the deviations of individual results from the mean, providing insight into the consistency of the method.

$$S = \sqrt{\frac{1}{n-1}\sum_{i-1}^{n}(x_i - \bar{x})^2}$$

Standard deviation has the same units as the property being measured.

The square of standard deviation is called variance (S^2). Relative standard deviation is the standard deviation expressed as a fraction of the mean, i.e., S/x. It is sometimes multiplied by 100 and expressed as a percent relative standard deviation. It becomes a more reliable expression of precision.

% Relative standard deviation = S x 100 / x

d) Accuracy

Accuracy is usually defined as the difference between the mean x**** of the set of results and the true or accurate value for the quantity measured. The difference between the real value and the results (or mean) is what the IUPAC refers to as accuracy. There are two ways to evaluate the accuracy of analytical methods: the absolute method and the comparative technique. The easiest way to report accuracy is as a percentage bias, which is determined using the expression.

$$\%Bias = \frac{(\text{measured value} - \text{true value}) \times 100}{\text{true value}}$$

The accuracy of the analytical method at each concentration is then determined by comparing the nominal and measured concentrations of the analytes in the spiked drug-free matrix sampler.

e) Limit of detection (LOD)

The concentration that results in an instrument signal that differs significantly from the blank is known as the analytical method's limit of detection (LOD). The IUPAC methodology use the standard deviation of the intercept (Sa) when employing spectroscopic techniques or other approaches that depend on a calibration curve for quantitative measurements. This is

connected to the slope, b, and LOD of the calibration curve by LOD = 3 Sa/ b

f) Limit of quantitation (LOQ)

The concentration that can be consistently measured with a particular degree of accuracy and precision is known as the limit of quantification, or LOQ. The limit of quantitation (LOQ) is the analyte concentration required to achieve a signal-to-noise ratio of 10. LOQ = 10 Sa/ b.

g) Ruggedness

Approach The reproducibility of results when the method is used in real-world scenarios is known as ruggedness. This covers various analysts, labs, columns, equipment, and sources of chemicals, solvents, reagents, etc. When a method is originally devised, its ruggedness may not be known, but later application of the approach provides insight.

h) Robustness

According to ICH, robustness is an analytical method's capacity to withstand minor, intentional adjustments to its parameters. It ensures the reliability of the method under varied conditions. Parameters such as mobile phase pH, temperature, organic solvent strength, and buffer concentration are typically varied to assess robustness. The method's stability is confirmed by evaluating changes in chromatographic characteristics under these altered conditions.

i) System suitability

To make sure an analytical method consistently yields findings with sufficient accuracy and precision, system suitability tests are carried out. Usually, these tests are created following the development and validation of the technique. Prior to analysis, the USP (2000) states that some parameters can be used to evaluate the system's appropriateness. The method's verified performance serves as the basis for the criteria. Retention times, for example, are frequently set within ±3 standard deviations (SD) of the values recorded during validation if they are included in the criterion 19-21

LITERATURE REVIEW

V.C.Yeligar, et. al: The goal of the study was to employ a simultaneous equation technique to design and validate a spectrophotometric method for the simultaneous measurement of quercetin and melatonin in a combination dose form. Melatonin's absorbance was measured at 276 nm, while quercetin's was recorded at 372 nm. The method followed ICH validation

guidelines, showing linearity in the 2–10 µg/ml range for both compounds. Correlation coefficients were 0.997 for melatonin and 0.993 for quercetin. The percentage estimation from the liposome formulation was 99.5% for melatonin and 99.75% for quercetin, with standard deviations below 2 (Veerendra C. Yeligar M.A. Rajmane et.al, 2017) 22

Gulcin ARSLAN AZİZOĞLU et al: A HPLC technique was developed and validated for the simultaneous determination of Melatonin and Octyl Methoxycinnamate in combined pharmaceutical and cosmetic formulations. Melatonin is valued for its antioxidant and photoprotective properties, making it suitable for sunscreen use, while Octyl Methoxycinnamate is a common UV filter approved up to 7.5% by the FDA. The study also involved the preparation of a microemulsion containing both compounds, which was characterized for droplet size, pH, and viscosity, demonstrating the method's applicability in real formulations. (Gulcin ARSLAN AZİZOĞLU et al, 2017) 23.

Mukthinuthalapati Mathrusri Annapurna et. al. (2016): For the simultaneous measurement of Lornoxicam and Paracetamol in tablet dose form, two straightforward and verified spectrophotometric techniques were created. While the second method (Q-Analysis) made use of the isoabsorptive point and the absorption maximum of one medication, the first method employed the simultaneous equation approach based on the absorption maxima of both medicines. In the concentration range of 0.1–40 µg/ml, both medications complied with Beer's law, demonstrating the precision and suitability of the techniques for routine analysis. (Mukthinuthalapati Mathrusri Annapurna et. al. (2016).24

Kala Shwetha, Singh Anita et. al(2016): This paper provides a review of analytical method validation, focusing on the development and validation processes for pharmaceutical drugs. The main objective is to outline the importance of method validation in pharmaceutical analysis and to summarize the key steps involved, ensuring the reliability, accuracy, and consistency of analytical methods used for drug evaluation.

Lalitha. KG et. al: Using a simultaneous equation approach, a spectrophotometric method was created and verified for the simultaneous determination of zolpidem and melatonin in a combination tablet dose form. Melatonin's absorbance was measured at 277 nm, while zolpidem's was recorded at 311 nm. The method followed ICH guidelines and showed linearity in the ranges of 3–10.5 µg/ml for melatonin and 5–17.5 µg/ml for zolpidem, with correlation coefficients of 0.9940 and 0.9969, respectively. The percentage estimation from

tablets was 101.80% for melatonin and 101.70% for zolpidem, with standard deviations below 2, confirming the method's accuracy, precision, and suitability for routine analysis. (Lalitha. KG et.al, 2014) 27

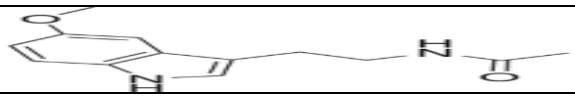
M.V.N.L. Chaitanya (2011): A comprehensive review on insomnia and its treatment was presented, including a brief introduction, classification, and causes of the condition. The review covered both non-pharmacological and pharmacological treatment approaches. Under pharmacological treatment, both allopathic and herbal medications were discussed. Additionally, various drugs were listed along with their generic and brand names, therapeutic dosages, and major side effects, providing a well-rounded overview of insomnia management.

Sacide Altınöz et.al: For drug dissolution investigations and the simultaneous measurement of amlodipine and valsartan in combination dose forms, a validated HPLC method was created. Using a C18 column and a mobile phase consisting of phosphate buffer (pH 3.6), acetonitrile, and methanol (46:44:10 v/v/v) at a flow rate of 1 mL/min, separation was accumpsised

DRUG PROFILE

4.1 MELATONIN

GENERAL DATA **Table no.2: Melatonin profile**

Drug	**Profile**
Synonym	5-methoxy-N-acetyltryptamine
Chemical Structure	
Drug Bank Code	DB01065
IUPAC	N-[2-(5-methoxy-1H-indol-3-yl)ethyl]acetamide
Molecular Framework	Aromatic hetero polycyclic compounds
Molecular formula	C13H16N2O2
Molecular weight	232.2783 g/mol
Monoisotopic	232.121177766
Physico Chemical Properties	
Appearance	White crystals or fine white powder.
Solubility	Soluble in water.
Melting Point	117 °C
PKa	Strongest Basic :15.8 Strongest Acidic : 1.6
LogP	1.6

4.1.1 Indication

The FDA has designated melatonin as an orphan medicine since it is commonly used orally to treat problems like jet lag, sleeplessness, shift-work disorder, and circadian rhythm disturbances, particularly in blind people. Strong evidence supports its effectiveness in these areas. It is also beneficial for sleep-wake disturbances in children and adolescents with developmental or neurological disorders such as autism, cerebral palsy, and intellectual disabilities, helping reduce sleep onset time. Melatonin may also assist with secondary insomnia and shows potential for treating conditions like benzodiazepine and nicotine withdrawal, cluster headaches, delayed sleep phase syndrome, preoperative anxiety, prostate cancer (with IL-2), sunburn (topically), tardive dyskinesia, and cancer or chemotherapy-related thrombocytopenia.

4.1.2 PHARMACOLOGY

4.1.2.1 Mechanism of action: Tryptophan derivative melatonin works by attaching itself to melatonin receptors MT1 and MT2, which are involved in a number of physiological functions. Melatonin inhibits adenylate cyclase and lowers cAMP synthesis upon binding. It also activates phospholipase C, which causes the release of arachidonate. The central nervous system (CNS) and peripheral organs such as the liver, hippocampus, hypothalamus, retina, and immune system are home to a large number of MT1 receptors. The central nervous system (CNS) and other tissues, including the heart, lungs, and reproductive organs, contain MT2 receptors. While MT2 activation also influences cGMP synthesis and may increase protein kinase C (PKC) activity, MT1 activation mainly decreases cAMP and protein kinase A (PKA) activity. Both receptor pathways influence ion channel activity and intracellular signaling, although the complete mechanisms remain not fully understood.

4.1.2.2 Pharmacodynamics

The pineal gland typically produces the hormone melatonin, which is then released into the bloodstream. Melatonin is made using L-tryptophan, an important amino acid, as a precursor. It makes the circadian rhythm—also referred to as sleep-wake cycles—easier to regulate. Darkness stimulates the generation of melatonin, while light inhibits it. Because it raises melatonin levels, which promote sleep, the medication is used to treat jet lag and insomnia. Because of their distinct pharmacologies and roles within the SCN, MT1 and MT2 receptors may be a target for the treatment of circadian and non-circadian sleep disorders. The 24-hour

cycle regulates a wide range of bodily processes from immunological responses to sleep, is maintained by SCN

4.1.2.3 Absorption: Melatonin's bioavailability and absorption vary greatly.

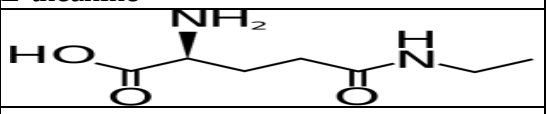
4.1.2.4 Metabolism

converted by the liver into a minimum of 14 recognized metabolites (discovered in the urine of mice): N-acetyl serotonin sulphate, 2-oxomelatonin, 3-hydroxymelatonin, cyclic melatonin, cyclic N-acetyl serotonin glucuronide, cyclic melatonin, cyclic melatonin, Five-hydroxyindole-3- acetaldehyde, 6-hydroxymelatonin glucuronide, 6-hydroxymelatonin sulphate, 6- hydroxy melatonin Di-hydroxy melatonin, 6-hydroxymelatonin, and its glucuronide conjugate 6- hydroxy melatonin glucuronide makes up 68% of the total melatonin metabolites found in mouse urine.

4.1.2.4 Half Life: 35 to 50 minutes[38,3]

4.2 THEANINE

GENERAL DATA Table no.3: L-Theanine Profile

Synonym	L-theanine
Chemical Structure	
Drug Bank Code	DB12444
IUPAC	(2S)-2-amino-4-(ethyl-C-hydroxy carbon imidoyl) butanoic acid
Molecular Framework	Aliphatic acyclic compounds
Molecular formula	C7H14N2O3
Molecular weight	174.1977 g/mol
Monoisotopic	174.100442324
Physico Chemical Properties	
Appearance	White crystals or fine white powder.
Solubility	Soluble in water.
Melting Point	174.20 °C
pK	Strongest Acidic - 2.1 Strongest Basic - 10.01
logP	-2.5

4.2.1 Pharmacology

4.2.1.1 Mechanism of action: One important amino acid that is only present in green tea is L-theanine (N-ethyl-L-glutamine), also known as theanine. Scientific studies on the pharmacology, kainate, and NMDA receptors of L-theanine have been prompted by its historical reports as a calming agent. Furthermore, in animal models, it has been

demonstrated to have neuroprotective benefits, presumably as a result of its antagonistic actions on group 1 methyl atrophic glutamate receptors. Animal behaveioural research points to enhanced memory and learning.

4.2.1.2 Pharmacodynamics

Theanine, structurally similar to glutamate, binds to ionotropic glutamate receptors such as AMPA, kainate, and to a lesser extent, NMDA receptors, though with lower affinity. It also blocks the reuptake of glutamine and glutamate. Theanine contributes to the umami taste by activating the T1R1 + T1R3 taste receptors. In the brain, it influences neurotransmitter levels by increasing serotonin, dopamine, GABA, and glycine, as well as neurotrophic factors like BDNF and NGF. However, its effect on serotonin remains debated, with conflicting findings in studies. Additionally, theanine may help reduce glutamate excitotoxicity and has shown to lower 5-hydroxyindole levels in hypertensive mice.

4.2.1.3 Absorption: According to research on animals, L-theanine seems to penetrate the blood-brain barrier and be absorbed from the small intestine by a sodium-coupled active transport mechanism. Rat experiments have revealed that the D-enantiomer of theanine may reduce L-theanine absorption.

4.2.1.4 Metabolism: Glutamate progressively generated in an in vitro medium containing theanine and glutaminase, indicating that theanine and glutaminase responded. Additionally, the reaction between theanine and gamma-glutamyl transpeptidase (gamma GTP) boosted the synthesis of glutamate, indicating that gamma-GTP converted theanine to glutamate. The liver's gamma-GTP hydrolysis and rearrangement process is thought to be the mechanism of theanine metabolism. Specifically, it is proposed that theanine-induced activity depends on the metabolism of theanine, which is mediated by glutaminase and gamma-GTP, as well as the rise in glutamine-mediated GSH.

4.2.1.5 Half-life: 0.5-1hour (healthy subjects, delayed-release capsule);3 hours (hepatic impairment)[40,43]

MATERIALS AND METHODS

6.1 MATERIAL

Table no. 4: List of Material.

Sr.No.	Name Of Materials	Supplier
1	Melatonin	Swapnroop Drug and Pharmaceutical, Aurangabad
2	L-Theanine	Swapanroop Drug and Pharmaceutical, Aurangabad
3	Meltofast Tablet	Medlife , Mumbai
4	Methanol (HPLC Grade)	Merck , Hyderabad
5	Buffer (KH2PO4) HPLC Grade	Merck , Hyderabad
6	Acetonitrile (HPLC Grade)	Merck, Hyderabad

Equipment

Table no. 5: List of Equipment.

Sr. No.	Name Of Equipment	Make
1	High Performance Liquid Chromatography	Waters 2695 (Compact system consisting Inertsil-C18 ODS Column and PDA detector)
2	UV-Visible Spectrophotometer	Systronics 119
3	FTIR Spectrophotometer	Agilent Technologies, Carry 630 FTIR
4	Sonicator	FAST CLEAN
5	Electronic Balance	SARTORIOUS
6	Melting Point	Esico International

6.2 TECHNIQUE DEVELOPMENT FOR HPLC

Based on the literature analysis, the aim of this work was to enhance the assay process for the simultaneous detection of L-theanine and melatonin. Thus, the aforementioned trials illustrate the optimization process.

6.3.1 Trial: 1

6.3.1.1 Mobile Phase: Degassed Acetonitrile 100%.

6.3.1.2 Preparation of Standard Solution

To get 1000 ppms, weigh out 10 mg of the drugs L-theanine and melatonin, dissolve them in 10 ml of mobile phase, and then sonicate each solution for 20 minutes. Next, dilute 1 ml of each solution in a 10 ml volumetric flask with 10 ml of mobile phase.

6.3.1.3 Chromatographic Conditions

Flow rate	: 1.0ml/min
Column	: Inertsil - C18, ODS column
Detector wavelength	: 278nm
Column température	: Ambiant
Injection volume	: 20µl

Run time	: 10min
Retention time	: 3.8min for Melatonin and 4.1 for L-Theanine.

6.3.1.4 Observation: Two peaks are combined rather than entirely split apart. Fig. 1 displayed the chromatogram result from trial 1.

6.3.2 Trail: 2

6.3.2.1 Mobile Phase: Methanol and degassed acetonitrile in a 90:10 V/V ratio.

6.3.2.2 Preparation of Standard Solution

In two separate 10 ml volumetric flasks, 10 mg of the medications melatonin and L-theanine should be weighed out, dissolved in 10 ml of mobile phase, and then sonicated for 20 minutes to reach 1000 ppms. Next, dilute 1 ml of each solution to 10 ml using mobile phase.

6.3.2.3 Chromatographic Conditions.

Flow rate	: 1ml/min
Column	: Inertsil -C18, BDS column
Detector wavelength	: 278nm
Column temp	: Ambiant
Injection volume	: 20µl
Run time	: 10min
Retention time	: 3.7 min for Melatonin and 4.0 min for L-Theanine.

6.3.2.4 Observation: The two peaks are separated completely but peak shapes are not good. The trial 2 chromatogram result was shown in Fig:2.

6.3.3 Trail: 3

6.3.3.1Mobile Phase: Degassed Acetonitrile and Methanol in the ratio of 80:20 V/V.

6.3.3.2 Preparation of Standard Solution

To get 1000 ppms, weigh out 10 mg of the drugs L-theanine and melatonin, dissolve them in 10 ml of mobile phase, and then sonicate each solution for 20 minutes. From each solution, one milli litre was extracted and diluted with mobile phase to yield ten milli litres.

6.3.3.3 Chromatographic Conditions

Flow rate	: 1.0ml/min
Column	: Inertsil - C18, BDS column
Detector wavelength	: 278nm

Column temp	: Ambient
Injection volume	: 20µl
Run time	: 10min
Retention time	: 1.7 min for Melatonin and 2.1 min for L-Theanine

6.3.3.4 Observation: L-Theanine got peak fronting and base line between two peaks is not straight. The trial 3chromatogram result was shown in Fig: 3.

6.4 OPTIMIZED METHOD

6.4.1 Mobile Phase: Methanol and buffer were degassed at a 60:40 V/V ratio.

6.4.2 Preparation of(KH2PO4 0.1M) buffer: In a beaker with 1000 millilitres of distilled water, weigh 3.8954 grammes of di-sodium hydrogen phosphate and 3.4023 grammes of potassium dihydrogen phosphate, then fully dissolve them. After using orthophosphoric acid to correct the pH, the mixture is filtered through a 0.45µm membrane filter.

6.4.3 Preparation of stock solution

Reference solution: In two 100.0 mL volumetric flasks, 20.0 mg of precisely weighed melatonin and 220.0 mg of L-theanine were combined in the mobile phase. After that, the mixture was sonicated for 20 minutes. Add the mobile phase and sonicate for 10 minutes after adding 10.0 mL of each of the previously listed solutions to a 50.0 mL volumetric flask.

6.4.4 Preparation of stock solution

Reference solution: In two 100.0 mL volumetric flasks, 20.0 mg of precisely weighed melatonin and 220.0 mg of L-theanine were combined in the mobile phase. After that, the mixture was sonicated for 20 minutes. Put 10.0 mL of each of the previously mentioned solutions in a 50.0 mL volumetric flask, then add the mobile phase and sonicate for 10 minutes.

6.4.5 Preparation of working standard solution

For both drugs, stock solutions containing 20 ppm to 80 ppm of melatonin and L-theanine were prepared. After that, they were filtered through a 0.45µ membrane and sonicated.

6.4.6 Preparation of Marketed formulation Drug Solution

Using a pestle and mortar, crush one tablet to its average weight, then weigh the precise equivalent weight of powder that includes 3 mg of MEL and 100 mg of THE, respectively. To

reach final concentrations of 30 µg/mL for MEL and 1000 µg/mL for THE in combined form, the aforementioned powder is dissolved in 100 ml of mobile phase in a volumetric flask and sonicated for 20 minutes. Additionally, 1 ml of sample solution is dissolved in 10 mL of volumetric flask, and the volume is adjusted using mobile phase.

6.4.7 Optimized chromatographic conditions

Table no. 6: Optimized chromatographic conditions.

Parameters	Method
Stationary phase (column)	Inertsil -ODS C18(250 x 4.6 mm, 5 µ)
Mobile Phase	Methanol : Buffer (60:40)
Flow rate (ml/min)	1.0 ml/min
Run time (minutes)	10 min
Column temperature (°C)	Ambient
Volume of injection loop (ml)	20
Detection wavelength (nm)	278nm
Drug RT (min)	4.250min for Melatonin and 5.995 for L-Theanine.

6.1 METHOD VALIDATION

6.5.1 SYSTEM SUITABILITY

In compliance with the test protocol, a standard solution was created using melatonin and L-theanine working standards and subsequently injected five times into the HPLC apparatus. By computing the percentage RSD from five replicate injections for L-theanine and melatonin, as well as retention periods and peak regions, the system appropriateness parameters were assessed from standard chromatograms.

6.5.1.1 ACCEPTANCE CRITERIA

6.5.1.1.1 For five replicate injections of each standard solution, the percentage RSD for the primary peak retention times should not exceed 2.0%.

6.5.1.1.2 The number of theoretical plates (N) for the Melatonin and L-Theanine peaks is NLT 3000

6.5.1.1.3 The tailing factor (T) for the melatonin

6.5.1.2OBSERVATION

It was discovered that the percentage RSD for peak areas and retention times was within the acceptable range. Refer to table 1 in figures 6–10.

6.5.2 SPECIFICITY

6.5.2.1 Melatonin and L-Theanine

The chromatographic system is filled with solutions of the standard and sample that were made in accordance with the test procedure

6.5.2.2 ACCEPTENCE CRITERIA

The standard and sample chromatograms need to be almost comparable in terms of retention time.

6.5.2.3 OBSERVATION

Both the Standard and Sample chromatograms had the same retention period and were identical. As illustrated in Figures 12 and 13.

6.5.3 PRECISION

6.5.3.1 REPEATABILITY

6.5.3.1.1 System precision: Standard solution prepared as per test method and injected five times.

6.5.3.1.2 Method precision: Prepared six sample preparations individually using single test method.

6.5.3.2 ACCEPTANCE CRITERIA

The % relative standard deviation of individual Melatonin and L-Theanine, from the six units should be not more than 2.0%.

The individual assays of Melatonin and L-Theanine should be not less than 98% and not more than 102.0%.

6.5.3.3 OBSERVATION

The test method's accuracy is demonstrated by the test results. For information on system and method precision, see tables 2 and 3.

6.5.4 ACCURACY (RECOVERY)

An investigation into accuracy was carried out. To determine the concentration of Melatonin and L Theanine equal to 50%, 100%, and 150% of the labelled amount according to the test procedure, the drug assay was carried out in triplicate using the same amount of each of the

two substances in each volumetric flask for each spike level. Melatonin and L-theanine recovery rates were averaged.

6.5.4.1 ACCEPTANCE CRITERIA

For both medications independently, the mean percentage recovery of L-theanine and melatonin at each spike level should be at least 98.0% and no more than 102.0%.

6.5.4.2 OBSERVATION

$$\% \text{ Recovery} = \frac{\text{Amount found}}{\text{Amount added}} \times 100$$

6.5.5 LINEARITY OF TEST METHOD

The Series of solutions are prepared using Melatonin and L-Theanine working standards.

6.5.5.1CRITERIA

Correlation Coefficient should be not less than 0.9990.

% of y- Intercept should be ±2.0.

% of RSD for level 1 and Level 6 should be not more than 2.0%.

6.5.5.2 OBSERVATION

The linear fit of the system was illustrated graphically. The results are presented in table 6

6.5.6 RUGGEDNESS OF TEST METHOD

6.5.6.1 System to system variability

A study of system-to-system variability was carried out on various HPLC systems at various times and under comparable circumstances. Each of the six produced samples was examined using the test procedure. The assay test procedure is robust for system-to-system variability, according to a comparison of the data from two distinct HPLC systems.

6.5.6.2 ACCEPTANCE CRITERIA

The relative standard deviations of L-theanine and melatonin from the six sample preparations shouldn't be more than 2.0%. Melatonin and L-theanine assay percentages should range from 98.0% to 102.0%.

6.5.6.3 OBSERVATION

The percentage RSD was found to be within the range. Refer to tables 3 and 7.

6.5.7 ROBUSTNESS

6.5.7.1 Effect of variation of flow rate

A study was carried out to ascertain the impact of flow rate fluctuation. Using flow rates of 1.0 and 1.2 milli litres per minute, the standard solution made in accordance with the test procedure was introduced into the HPLC system. After evaluation, the system suitability characteristics were determined to be within the range for flows of 1.0 and 1.2 millilitres per minute. All other peaks were separated by melatonin and L-theanine, and the retention durations matched those of the mobile phase with flow rates of 1.0 ml/min.

6.5.7.2 ACCEPTANCE CRITERIA

For melatonin and L-theanine standards, the tailing factor should be NMT 2.0 for flow variation.

6.5.7.3 OBSERVATION

The tailing factor for Melatonin and L-Theanine was found to be within the limits. As shown in table 10.(K. S. Patil et al, 2010, A.A.Vitale et al; 1996 and B. S Kuchekar et al; 2002)

6.5.7.4 LIMIT OF DETECTION AND QUANTITATION (LOD and LOQ)

It was discovered that the tailing factors for L-theanine and melatonin were within acceptable bounds. as displayed in Table 10.

(K. S. Patil et al, 2010, A.A. Vitale et al; 1996 and B. S Kuchekar et al;2002)

$$LOD = \frac{3.3\ \sigma}{S}$$

σ = standard deviation of the response

S = slope of the calibration curve of the analyte.

$$LOQ = \frac{10\ \sigma}{S}$$

σ = standard deviation of the response

S = slope of the calibration curve of the

6.6 FORMULA USED FOR CALCULATION

Formulae's used in calculation of Amount found, % Recovery and % RSD and % label claim in Experimental work.

1) **Amount Found** = ((Area-intercept))/slope

2) **%Recovery** = (Amount Recovered)/(Amount of std Added) x100

3) **%RSD** = (S.D.of Amount found)/(Mean of amount found) x100

4) **% label Claim** = (amount found)/(label claim)*100

RESULT AND DISCUSSION

7.1 IR SPECTRA

7.1.1 FTIR Spectra of MEL

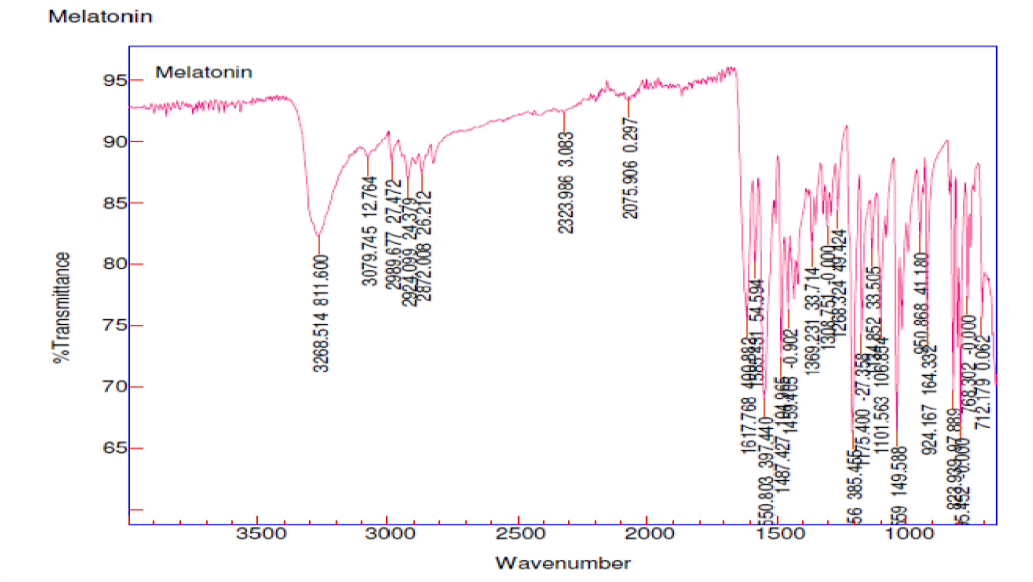


Fig.no.1: Melatonin IR spectra.

The FTIR spectrum verifies the presence of key functional groups in melatonin, such as alkyl bonds (- CH_3/-CH_2), amides (-CONH-), methyl (-OCH_3), and aromatic rings (indole structure). These results are consistent with N-acetyl-5-methoxytryptamine, the molecular component of melatonin. The FTIR spectrum helps verify the authenticity and purity of melatonin by comparing its vibrational fingerprints to known functional groups.

7.1.2 FTIR Spectra of THE

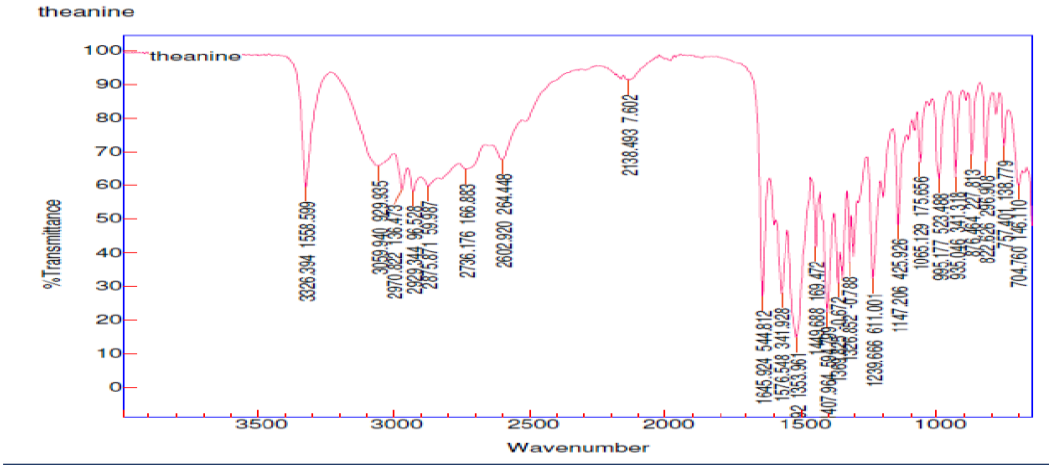


Fig.no.2: L- Theanine IR spectra.

The FTIR spectrum of theanine confirms the presence of: carboxylate group stretch (-COO⁻ ~1558 cm^{-1}), aliphatic chains (lengths 3050–2850 cm^{-1} from C to H), C–N and C–O links, and the N–H stretch, which is roughly 3326 cm^{-1}. These functional groups align with the structure of L-theanine (γ glutamyl ethyl amide), an amino acid analogue found in tea. The distinctive functional group vibrations in the spectrum contribute to its distinctiveness.

7.2 Method Development by UV spectrophotometer

7.2.1 Selection of Wavelength

Scan a standard solution in a UV spectrophotometer set to spectrum mode between 200 and 400 nm of MEL and THE using diluents as a blank. 278 nm is the usual wavelength.

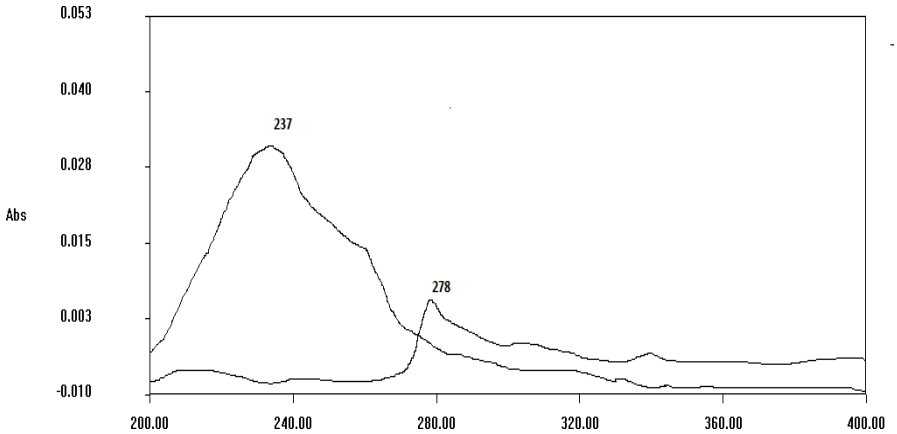


Fig. no. 3: Selection of UV Wavelength.

7.2.2 Linearity: To prepare standard stock solution, a series of solutions were prepared a Concentration range 4-24µg/ml.

Table no. 7: Observation table of MEL.

Concentration(µg/ml)	Absorbance	Correlation Coefficient
4	0.071	
8	0.141	
12	0.211	0.999
16	0.283	
20	0.355	
24	0.422	

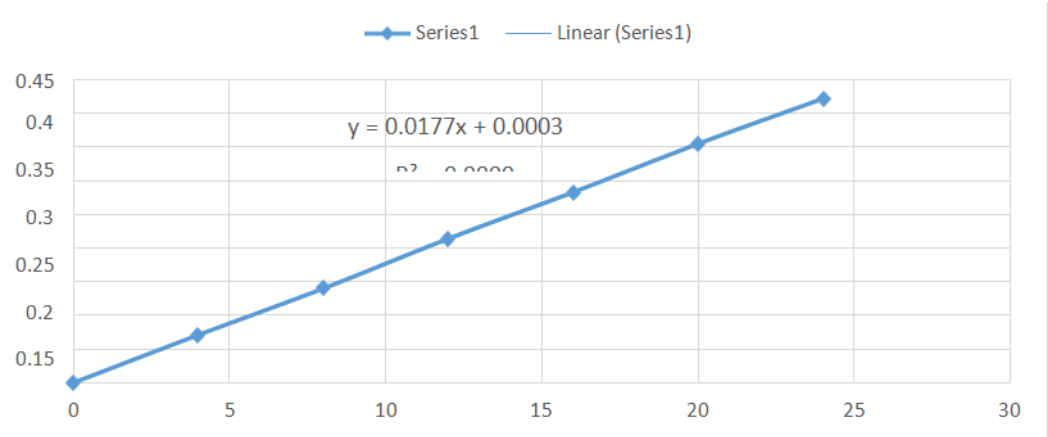


Fig. No.4: Linearity graph of MEL (Cons. Vs Abs)

Table no. 8: Observation table of THE.

Concentration(µg/ml)	Absorbance	Correlation Coefficient
4	0.071	
8	0.141	
12	0.211	0.999
16	0.283	
20	0.355	
24	0.422	

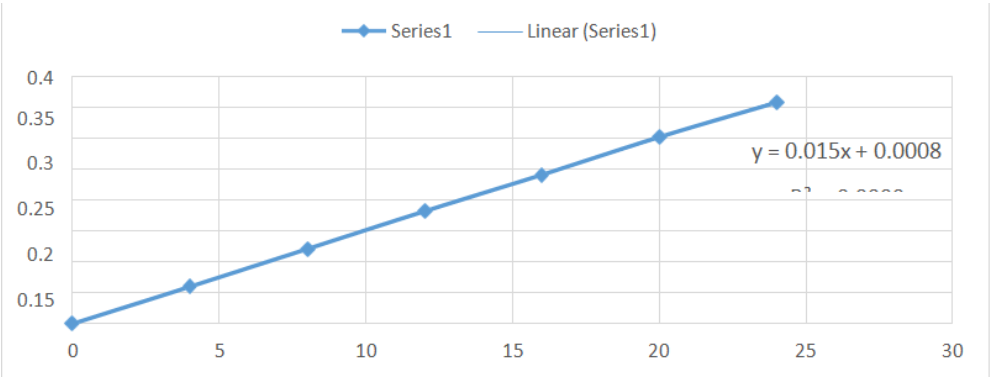


Fig. No.4: Linearity graph of THE (Cons. Vs Abs).

7.2.3 PRECISION

Five replicates of each solution were prepared for the intra-day and inter-day experiments, and the absorbance was measured three times on the same day and three more days. To evaluate repeatability and reproducibility, the study's findings are expressed as a percentage of RSD.

TABLE no. 9: Data of Analyst 1.

Sr. No:	Absorbance of MEL	Absorbance of THE
1	0.181	0.211
2	0.181	0.212
3	0.182	0.211
4	0.181	0.210
5	0.183	0.212
Mean	0.181	0.211
SD	0.0008	0.0008
% RSD	0.492	0.396

TABLE no. 10: Data of Analyst 2

Sr. No:	Absorbance MEL	Absorbance THE
1	0.183	0.212
2	0.184	0.212
3	0.183	0.214
4	0.183	0.213
5	0.182	0.213
Mean	0.183	0.212
SD	0.0007	0.0008
% RSD	0.386	0.393

7.2.4 Limit of detection (LOD) and Limit of quantitation (LOQ)

The detection limit was determined using the calibration curve's slope and the response's standard deviation.

Melatonin

$$LOD = \frac{3.3 * SD}{Slope}$$

$$= \frac{3.3 * 0.0008}{0.017}$$

= 0.1624

$$LOQ = \frac{10 * SD}{}$$

Slope

10*0.0008

= ---------- 0.017

= 0.492

L-Theanine

3.3*SD LOD -----

Slope

3.3*0.0008

= 0.017 ----------

= 0.1624

10*SD LOQ = ----------

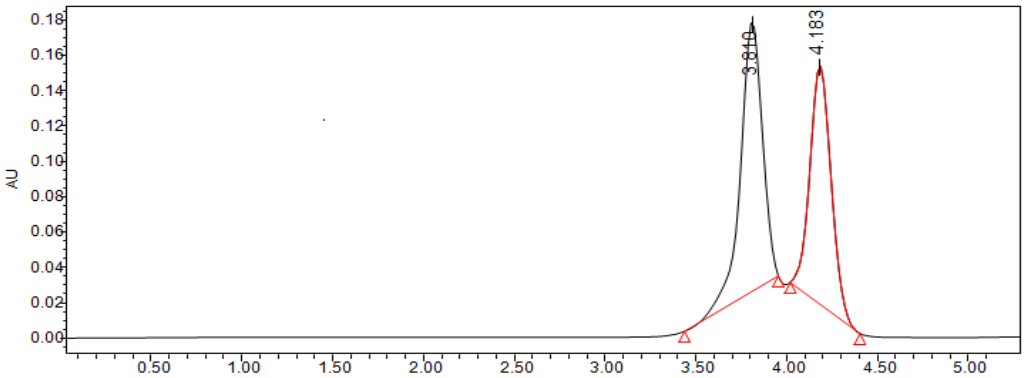
Slope

10*0.0008

= ---------- 0.017

= 0.492

7.2 Method Development by RP-HPLC



Figno.6: Chromatogram of Trial 1

7.3.2 VALIDATION DATA

A. System Suitability

Table no. 11 (a): Data of System Suitability for MEL.

Injection	RT	Peak Area	USP Plate count	USP Tailing
1	4.253	1139272	5890.964069	1.238915
2	4.248	1140892	5915.423628	1.230637
3	4.249	1136301	5934.796986	1.240858

4	4.250	1141067	5976.253744	1.238995
5	4.251	1136024	5953.814152	1.241073
Mean	4.2502	1138711	5934.251	1.236496
SD	0.001924	57540.015	-------	-------
% RSD	0.045258	0.213538	-------	-------

Table no: 11 (b): Data of System Suitability for THE.

Injection	RT	Peak Area	USP Plate count	USP Tailing
1	6.000	797564	8676.113795	1.099100
2	5.996	795138	8803.641669	1.103929
3	5.998	795685	8616.937115	1.111477
4	6.002	800569	8820.182543	1.117660
5	6.002	797049	8735.115629	1.119004
Mean	5.9996	797201	8730.398	1.110234
SD	0.002608	2124.413	-------	-------
% RSD	0.043464	0.266484	-------	-------

A. Precision

a) Repeatability

(i) System precision

Table no. 12: Data of Repeatability (System precision) for MEL and THE.

	Injection	Peak Areas of Melatonin	%Assay	Peak Areas of L-Theanine	%Assay
Concentration 40ppm	1	1146923	99.65	801690	98.84
	2	1143596	99.08	797631	99.69
	3	1158293	99.98	805783	100.05
	4	1147283	100.04	801496	101.11
	5	1152490	100.16	806432	100.96
Statistical Analysis	**Mean**	1149717	99.78	802606.4	100.13
	SD	5754.015	0.435569	3590.034	0.937203
	% RSD	0.500472	0.43652	0.447297	0.935987

(ii) Method precision

Table no. 13: Data of Repeatability (Method precision) for Melatonin and L-Theanine.

	Injection	Peak Areas of MEL	%Assay	Peak Areas of THE	%Assay
Concentration 40ppm	1	1152293	99.55	805783	99.85
	2	1146923	99.88	801690	99.96
	3	1147283	99.40	801496	100.53
	4	1152490	99.56	806432	100.30
	5	1139272	99.85	797564	100.08
	6	1147283	99.40	801496	100.53
Statistical Analysis	**Mean**	1147591	99.67	801593	100.20
	SD	4815.615	0.250093	3262.714	0.290477
	% RSD	0.419628	0.250913	0.406614	0.289873

(i) Intermediate precision

For Analyst 1 refer: Table no. 12

Table no. 14: Data of Intermediate precision (Analyst 2) for Melatonin and L-Theanine.

	Injection	Peak Areas of Melatonin	%Assay	Peak Areas of L-Theanine	%Assay
Concentration 40ppm	1	1139272	98.80	797564	99.85
	2	1140892	99.54	795138	99.68
	3	1136601	99.98	795685	100.08
	4	1141067	100.02	800569	100.01
	5	1136024	101.08	797049	99.52
	6	1140892	99.54	795685	100.08
Statistical Analysis	**Mean**	1139125	99.82	796948.3	100.37
	SD	2281.417	0.755001	1998.386	0.337086
	% RSD	0.200278	0.756312	0.250755	0.337299

B. Accuracy (Recovery)

Table no. 15: (i) Data of Accuracy for MEL.

Concentration % of spiked level	Amount added (ppm)	Amount found (ppm)	% Recovery	Statistical Analysis of % Recovery	
50% Injection 1	20	19.85	99.25	**MEAN**	99.88
50% Injection 2	20	19.96	99.80		
50% Injection 3	20	20.12	100.6	**%RSD**	0.67
100 % Injection 1	40	39.74	99.35	**MEAN**	99.81
100 % Injection 2	40	40.08	100.2		
100% Injection 3	40	40.24	100.6	**%RSD**	0.399
150% Injection 1	60	59.04	98.40	**MEAN**	99.19
150% Injection 2	60	59.62	99.36		
150% Injection 3	60	59.89	99.81	**%RSD**	0.72

A. Linearity

Table no. 16: Data of Linearity Melatonin L-Theanine.

Concentration (ppm)	Average Area	Statistical Analysis MEL		Average Area	Statistical Analysis THE	
0	0	Slope	31282	0	Slope	20193
20	523467	y-Intercept	11218	412977	y-Intercept	1902
		Correlation			Correlation	

30	829544	Coefficient	0.999	605369	Coefficient	0.999
40	1139272			807564		
50	1448018			1007428		
60	1728926			1210925		
70	2089505			1409560		
80	2407574			1627087		

A. Ruggedness

(a) System to System variability: For system 1 Refer: Table no.12

Table no. 17: Data of system to system variability Melatonin and L-Theanine System-2.

S.NO.	Peak area	Assay % of MEL	Peak area	Assay % of THE
1	1146923	99.65	801690	98.84
2	1143596	99.08	797631	99.69
3	1158293	99.98	805783	100.05
4	1147283	100.04	801496	101.11
5	1152490	100.16	806432	100.96
6	1158293	99.98	800569	100.01
Mean	1151146	99.78	802266.8	100.11
%RSD	0.540725	0.43652	0.413454	0.838768

A. Robustness

Table no. 18: Data for Effect of variation in flow rate (Melatonin).

	Std Area	Tailing factor		Std Area	Tailing factor		Std Area	Tailing factor
Flow 0.8 ml	1139272	1.238915	**low 1.0 ml**	1146923	1.251658	**Flow 1.2 ml**	1152293	1.262464
	1140892	1.230637		1143596	1.245435		1146923	1.251658
	1136301	1.240858		1158293	1.262464		1147283	1..237018
	1141067	1.238995		1147283	1.237018		1152490	1.239010
	1136024	1.241073		1152490	1.239010		1139272	1.238915
Avg	1138711	1.236496	**Avg**	1149717	1.247117	**Avg**	1148852	1.245813
SD	2431.578	0.005254	**SD**	5754.015	0.010328	**SD**	7076.841	0.010984
%RSD	0.213538	0.424907	**%RSD**	0.500472	0.008282	**%RSD**	0.615992	0.00881712

Table no. 19: Data for Effect of variation in flow rate (L-Theanine).

	Std Area	Tailing factor		Std Area	Tailing factor		Std Area	Tailing factor
Flow 0.8 ml	797564	1.099100	**Flow 1.0 ml**	801690	1.122813	**Flow 1.l**	805783	1.121321
	795138	1.103929		797631	1.112181		801690	1.122813
	795685	1.111477		805783	1.121321		01496	1.124805
	800569	1.117660		801496	1.124805		806432	1.123373
	797049	1.119004		806432	1.123373		797564	1.099100
Avg	797201	1.110234	**Avg**	802606.4	1.120899	**Avg**	801593	1.118282
SD	2124.413	0.008622	**SD**	3590.034	0.00503	**SD**	3613.298	0.047969
%RSD	0.500472	0.77655	**%RSD**	0.447297	0.004488	**%RSD**	0.450203	0.965376

A. Analysis of Marketed Formulation

Table no. 20: Analysis of Meltofast tablet.

Formulation	Labeled claim (mg/tab)	Amount found (mg/tab)*	Average Percentage Label Claim*	SD	%RSD
Meltofast	Melatonin 3mg	3.3mg	101.8	0.0635	0.1559
	L-Theanine 100 mg	98.03mg	98.03	0.0624	0.6365

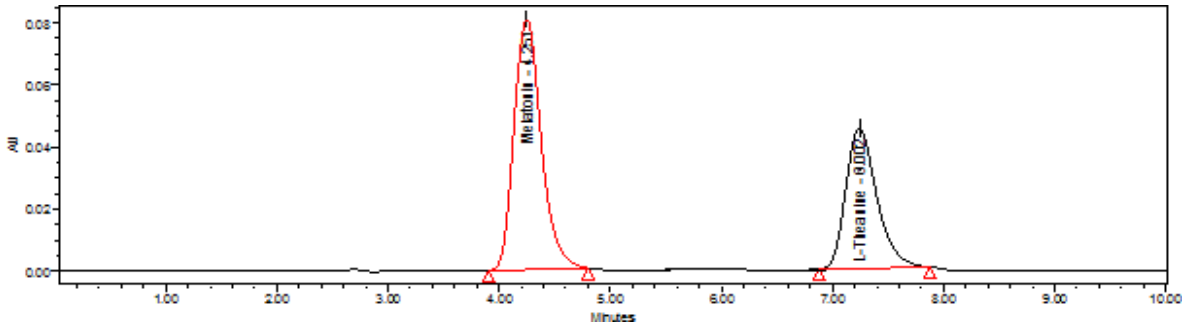


Fig. no. 36: Chromatograph of Meltofast Tablet.

Inference: Chromatograph of Meltofast Tablet RT of MEL (5.37min) and The (7.27Min)

7.3.3 LIMIT OF DETECTION AND LIMIT OF QUANTITATION (LOD and LOQ)

Melatonin

From the linearity plot the LOD and LOQ are calculated.

$$LOD = \frac{3.3\,\sigma}{S} = \frac{3.3 \times 2431.578}{31282} = 0.25$$

$$LOQ = \frac{10\,\sigma}{S} = \frac{10 \times 2431.578}{31282} = 0.77$$

L-Theanine

$$LOD = \frac{3.3\,\sigma}{S} = \frac{3.3 \times 2124.413}{20193} = 0.34$$

$$LOQ = \frac{10\,\sigma}{S} = \frac{10 \times 2124.413}{2019} = 1.05$$

CONCLUSION

An analytical method was developed for the simultaneous estimation of melatonin and L-theanine by evaluating key parameters. Maximum absorbance was observed at 237 nm for melatonin and 276 nm for L-theanine, with a common wavelength set at 278 nm. The method used a 20 µl injection volume and an Inertsil C18 ODS column, providing good peak shape.

A mobile phase of Methanol :Buffer (60:40) at a flow rate of 1.0 ml/min yielded symmetrical peaks and optimal resolution. The method showed good linearity over the range of 20–80 ppm with recovery between 98.0–101.5%. Detection limits were 0.25 µg/ml for melatonin and 0.34 µg/ml for L-theanine.

The method passed robustness and ruggedness tests, and both system and method precision were within acceptable limits, confirming its accuracy, reliability, and suitability for routine analysis.

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