'DEVELOPMENT AND VALIDATION OF UV SPECTROPHOTOMETRIC AND STABILITY INDICATING RP-HPLC ASSAY METHOD FOR AGOMELATINE IN BULK AND PHARMACEUTICAL FORMULATION ″

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By

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CERTIFICATE

This is to certify that the dissertation work entitled "Development and Validation of UV Spectrophotometric and Stability Indicating RP-HPLC Assay Method for Agomelatine in Bulk and Pharmaceutical Formulations" submitted by Mr. Jaykumar Pareshkumar Shah with Reg. No. (10MPH304) in partial fulfillment for the award of Master of Pharmacy in "Pharmaceutical Analysis" is a bonafide research work carried out by the candidate at PTC, ADL(Japan), Cadila Healthcare Ltd. This work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.

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DECLARATION

I hereby declare that the dissertation entitled "Development and Validation of UV Spectrophotometric and Stability Indicating RP-HPLC Assay Method for Agomelatine in Bulk and Pharmaceutical Formulations" is based on the original work carried out by me under the guidance of Dr. Priti J. Mehta, Head of the Department, Department of Pharmaceutical Analysis, Institute of Pharmacy, Nirma University and Mr. Vijayendrasingh Chauhan, Deputy Manager PTC, ADL(Japan), Cadila Healthcare Ltd. I also affirm that this work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.

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LIST OF ABBREVIATIONS

AGO	Agomelatine
API	Active pharmaceutical ingredient
API	Active Pharmaceutical Ingredients
Conc.	Concentration
°C	Degree Celsius
DPA	Degradation peak in acidic condition
DPB	Degradation peak in basic condition
g	Gram
GC	Gas chromatography
GR grade	Guaranteed reagent grade
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid/ Hydrochloride
HPLC	High performance liquid chromatography
ICH	International Conference on Harmonization
IR Spectra	Infrared Spectra
LC	Liquid chromatography
LOD	Limit of quantification
LOQ	Limit of detection
mg	Milligram
Min.	Minute
mL	Millilitre
NaOH	Sodium hydroxide
NLT	Not less than

NMT	Not more than
nm	Nanometres
OPA	Ortho-phosphoric acid
PDA	Photo diode array
RP-HPLC	Reverse phase high performance liquid chromatography
RSD	Relative standard deviation
RT	Retention time
S/N ratio	Signal to noise ration
SD	Standard deviation
SFC	Supercritical fluid chromatography
TLC	Thin layer chromatography
USP	United state pharmacopoeia
UV Spectra	Ultra violet spectra
v/v	Volume/volume
λ_{max}	Maximum wavelength
µ	Micro

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ABSTRACT

Agomelatine is a novel anti-depressant drug. The aim of the present work was to develop and validate an accurate, simple, rapid and cost effective UV-Visible specrophotometric and stability indicating RP-HPLC method for estimation of Agomelatine in bulk and in pharmaceutical dosage form. In UV-Visible spectrophotometric method estimation of Agomelatine was done on its λ_{max} 230nm using water: ACN (50:50) as a solvent. The calibration curve was found linear in the range of 0.5 – 4.0 µg/ml for Agomelatine (R^2= 0.9999). A sensitive stability indicating RP-HPLC method was developed with ZORBAX Eclipse XDB C_{18} column (150 mm x 4.6mm x 5µm) with optimized mobile phase containing Buffer (pH 3.0): ACN: TEA (62:38:0.1 %v/v/v). Detection of Agomelatine was carried out at 230nm. The linearity for Agomelatine was found in the range of 10 – 40 µg/mL (R^2= 0.9999). Other parameters for the developed methods were validated according to the ICH guidelines. High percentage recovery shows that there is no interference of excipients in the dosage form. Both the methods were applied on pharmaceutical dosage form. The proposed UV-Visible Spectrophotometric method was accurate, precise, sensitive and cost-effective that can be successfully applied to bulk drugs and pharmaceutical formulation. The stability indicating RP-HPLC assay method was also simple, accurate, precise and specific and has the ability to separate the drug Agomelatine, its degradation products.

CHAPTER 1

INTRODUCTION

1.0 ANALYTICAL CHEMISTRY[1-4]

Analytical chemistry is a branch of chemistry that deals with the separation, identification and determination of components in a sample. It is the science of making quantitative measurements, which requires background knowledge of chemical and physical concepts. To be efficient and effective, an analytical chemist must know the tools that are available to tackle a wide variety of problems. For this reason, analytical chemistry courses are often structured along the lines of the analytical methods (the tools-of-the-trade).

Understanding the analytical toolbox requires a scientist to understand the basic principles of the analytical techniques. With a fundamental understanding of analytical methods, a scientist faced with a difficult analytical problem can apply the most appropriate techniques(s). A fundamental understanding also makes it easier to identify when a particular problem cannot be solved by traditional methods, and gives an analyst the knowledge that is needed to develop creative approaches or new analytical methods.

Analytical Chemistry involves separating, identifying and determining the relative amounts of the components making up the sample. The qualitative analysis reveals the chemical identity of the sample while quantitative analysis gives the amount of one or more of the components present in numerical terms.

Pharmaceutical analysis deals not only with medicaments (drugs and their formulations) but also with their precursors i.e. with the raw material on which degree of purity and quality of medicament depends. The quality of the drug is determined after establishing its authenticity by testing its purity and the quality of pure substance in the drug and its formulations.

Quality is important in every product or service but it is vital in medicine as it involves life. Unlike ordinary consumer goods there can be no “second quality” in drugs. Quality control is a concept which strives to produce a perfect product by series of measures designed to prevent and eliminate errors at different stages of production.

1.1. Need for drug analysis:

The number of drugs introduced into the market is increasing every year. These drugs may be either new entities or partial structural modification of the existing one. Very often there is a time lag from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. This happens because of the possible uncertainties in the continuous and wider usage of these drugs, reports of new toxicities, development of patient resistance and introduction of better drugs by competitors. Under these conditions, standards and analytical procedures for these drugs may not be available in the pharmacopoeias. It becomes necessary, therefore to develop newer analytical methods for such drugs.

In brief, the reasons for the development of newer methods of drug analysis are:

- The drug or drug combination may not be official in any pharmacopoeias.
- A proper analytical procedure for the drug may not be available in the literature due to patent regulations.
- Analytical methods may not be available for the drug in the form of a formulation due to the interference caused by the formulation excipients.
- Analytical methods for the quantitation of a drug in biological fluids may not be available.
- Analytical methods for a drug in combination with other drugs may not be available.
- The existing analytical procedures may require expensive reagents and solvents. It may also involve cumbersome extraction and separation procedures and these may not be reliable.

Analytical techniques that are generally used for the drug analysis are biological and microbiological methods, radioactive methods, physical methods and miscellaneous techniques like conventional titrimetric, gravimetric and polarimetric methods.

The use of instrument is an exciting and fascinating part of chemical analysis that interacts with all areas of chemistry and with many other areas of pure and applied science. Analytical instrument plays an important role in the production and evaluation of new products, protection of consumers and environment; it also provides the lower detection limits required to assure safe food, water and air.

1.1.1 Advantages of instrumental methods:

- Small samples can be used
- High sensitivity is obtained
- Determination is very fast
- Complex samples can be handled easily

1.1.2 Limitations of instrumental methods:

- An initial or continuous calibration is required
- Sensitivity and accuracy depends on the instrument or the wet chemical method
- Cost of equipment is large
- Concentration range is limited
- Specialized training is needed
- Sizable space is required

1.1.3 Principle types of chemical instrumentation

1.1.3.1 Spectrometric techniques

- UV-Visible spectrophotometry
- Fluorescence and phosphorescence spectrophotometry.
- Atomic spectrometry (emission and absorption)
- Infrared spectrophotometry
- Raman spectroscopy
- X-Ray spectroscopy
- Radiochemical techniques including activation analysis
- Nuclear magnetic resonance spectroscopy
- Electron spin resonance spectroscopy

1.1.4.2 Electrochemical techniques

- Potentiometry
- Voltammetry
- Voltametric techniques
- Stripping techniques.

- Amperometric techniques
- Colorimetry
- Electrogravimetry
- Conductance techniques

1.1.4.3 Chromatographic techniques

- Gas chromatography
- High performance liquid chromatography
- Thin layer chromatography

1.1.4.4 Miscellaneous techniques

- Thermal analysis
- Mass spectrometry

1.1.4.5 Hyphenated techniques

- GC-MS (Gas chromatography – mass spectrometry)
- ICP-MS (Inductivity coupled plasma mass spectrometry)
- MS-MS (Mass spectrometry – mass spectrometry)
- GC-IR (Gas Chromatography – Infrared Spectroscopy)

1.2 UV-VISIBLE SPECTROSCOPY [3-4]

Spectrophotometers may employ a single beam or double beam optical system. In a single beam instrument, a beam of radiant energy from a stable source is allowed to pass through a reference medium and then readout is adjusted to a 100% transmittance or zero absorbance setting, either by mechanical, optical or electrical compensation. The beam is then passed through the sample to get photometric readings.

An absorption measurement with a single beam spectrophotometer thus involves three steps:

1. The indicator is first adjusted to zero transmittance or infinite absorbance in which no radiation strikes the detector.
2. Then the indicator is adjusted to 100% transmittance or zero absorbance, with the cell filled with solvent in the beam of the instrument.

3. Finally, the solvent is replaced by the solution under examination and transmittance or absorbance is noted directly from the indicator scale.

Double beam instruments, uses a beam splitting device to produce two optical beams. One beam passes through the reference cell, while the other passes through the sample cell. Thus the dispersed radiation in a double beam photometer is splited into two components, one passes through the sample solution and other through the solvent.

The double beam system components for any source fluctuations is amenable to automatic recording. It is also possible for double beam instruments to be used so that the reference beam monitors only the source and that both the reference and sample solutions could be measured alternately in the same optical path.

Single beam instruments are particularly used in the quantitative analysis that involves an absorption measurement at a single wavelength. For greater speed as well as convenience of measurement, the double beam instruments particularly useful for qualitative analysis, many absorbance measurements are to be made at several wavelengths.

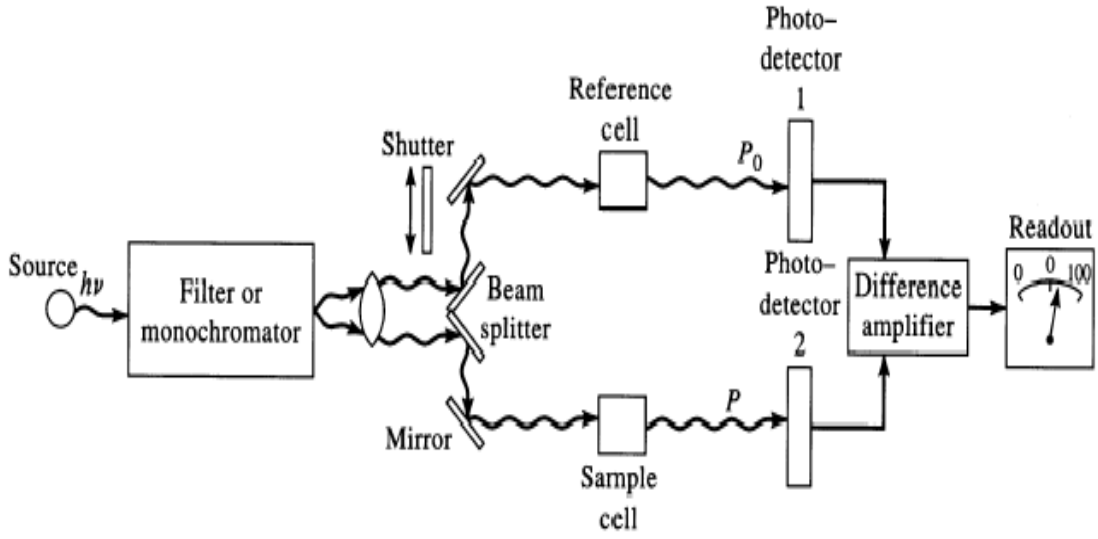


Fig 1.1: Double Beam UV Spectrophotometer

1.2.1 Principle of UV-Visible spectroscopy:

UV-Visible spectroscopy is based upon Beer-Lambert law. When a beam of light is passed through a transparent cell containing a solution of an absorbing substance,

reduction of the intensity of the light may occur and by measurement of decrease in the intensity of light, one can calculate the amount of absorbed light.

$$\mathbf{A = abc}$$

Here, A = absorbance

c = concentration

b = path length

a = absorptivity of the system.

Beer's law is applicable under the following conditions:

1. The incident radiation beam is monochromatic.
2. The incident radiation travels through equidistant parallel path through the sample.
3. There is no chemical reaction occurring followed by absorption of radiation energy.
4. The sample solution is homogenous and thus, there is no loss of radiant energy through scattering or by other reflection process.
5. Each molecule absorbs independently and is not affected by other molecule in solution (note: this may be not true for high solute concentration).

1.2.2 Method development by UV-Visible spectroscopy:

Estimation of drugs by spectrophotometric methods rarely involves samples containing only one absorbing species. Analysts have modified simple procedures in spectrophotometry to eliminate certain sources of interferences, or alternately to detect the amount of other absorbing species. As a result of which accurate determination one or all of the absorbing species and components is possible.

Solvent extraction procedures may be employed to separate the interferents if their contribution to the total absorbance cannot be calculated. These are particularly appropriate for acidic or basic drugs where state of ionizations determine their solvent partition behaviour. The judicious choice of pH of the aqueous medium and of immiscible solvent may effect the complete separation of interferentes from the

analyte, the concentration of which may be obtained by a single measurement of absorbance or extract containing the analyte.

1.2.3 UV-Visible spectrophotometry methods for single drug:

UV visible spectroscopy is generally used for quantitative determination of compounds that absorb UV and visible radiation. This determination is based on Beer's law, which is as follows:

$$\mathbf{A = \log_{10} 1/T = \log (Io/ I) = \varepsilon bc}$$

Where **ε** is the molar extinction coefficient, c is the concentration and b is the length of cell used in UV-Visible spectrophotometer. For development of an assay method to determine concentration of a substance in bulk drug or dosage from, wavelength of maximum absorption for the compound is selected. Then, the optical densities of compound are selected. Then, the optical densities of compound are measured for different concentrations. Graph is plotted between concentration v/s optical density. This plot may be used as calibration curve for determining the unknown concentration of solute. The solution of unknown strength is scanned in the spectrophotometer and its optical density is measured corresponding to this optical density, the concentration of unknown solution can be measured from calibration plot.

1.3 HPLC (HIGH PERFORMANCE LIQUID CHROMATOGRAPHY):

A variety of methods are available for analyzing pharmaceutical compounds; however, high performance liquid chromatography is currently the method of choice for the analysis of these compounds. High performance liquid chromatography is at times called high-performance liquid chromatography because it offers high performance over ambient pressure or low-pressure liquid chromatography[5].

HPLC must be able to resolve mixtures with a large number of similar analytes. HPLC chromatogram provides directly both qualitative and quantitative information: each compound in the mixture has its own elution time (the point at which the signal appears on the recorder or screen) under a given set of conditions; and both the area and height of each signal are proportional to the amount of the corresponding

substance[6]**.** Since the analytical technique is only one part of the overall analytical method. An analytical method may be thought of as consisting of five distinct parts:

- Defining the problem
- Taking the sample
- Sample pre-treatment
- Measurement
- Calculating, assessing and reporting the results[6]**.**

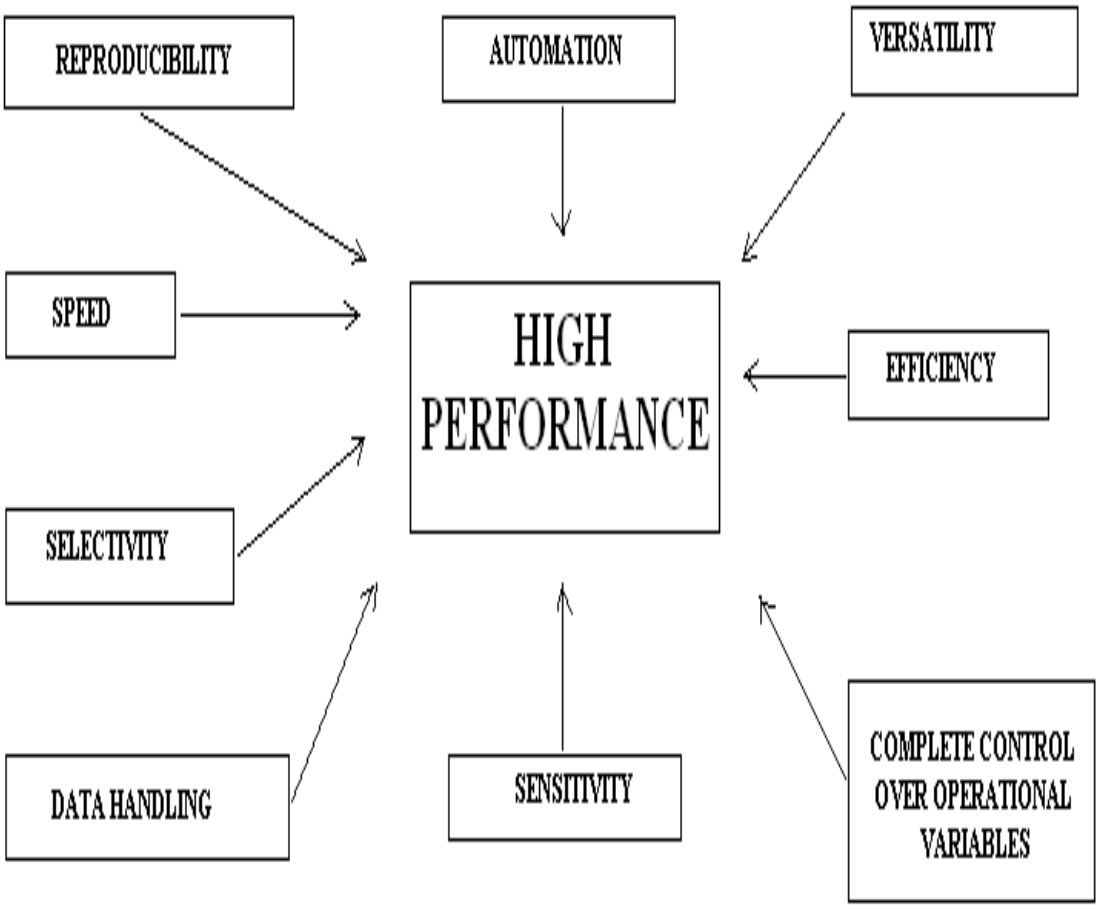


Fig. 1.2: Components of performance as defined by C. Horvath[7]

HPLC's virtue lies in its versatility. We can use it to separate compounds of molecular weights from 54 to 450,000 Daltons. Amounts of material to be detected can vary from picograms and nanograms (analytical scale) to micrograms and milligrams (semi-preparative scale) to multigrams (preparative scale). There are no requirements for volatile compounds or derivatives. Aqueous samples can be run directly after a simple filtration. Compounds with a wide polarity range can be analyzed in a single run[6]. Pharmacopoeial assay still rely quite heavily on direct UV-Visible spectroscopy but in industry detection by UV-Visible spectrophotometry is usually combined with a preliminary separation by HPLC[8].

HPLC offers numerous advantages as stated below:

- Capable of handling **macromolecules**
- Suitable for **pharmaceutical compounds**
- Efficient analysis of **labile natural products**
- Reliable handling of **inorganic or other ionic species**
- Dependable analysis of **biochemicals.**

1.3.1 Normal phase HPLC

This method separates analytes based on adsorption to a stationary surface chemistry and by polarity. It was one of the first kinds of HPLC that chemists developed. NP-HPLC uses a polar stationary phase and a non-polar, non-aqueous mobile phase, and works effectively for separating analytes readily soluble in non-polar solvents. Adsorption strengths increase with increased analyte polarity, and the interaction between the polar analyte and the polar stationary phase (relative to the mobile phase) increases the elution time. The interaction strength depends not only on the functional groups in the analyte molecule, but also on stearic factors.

1.3.2 Reversed phase HPLC

In this case, the column size is the same, but the silica is modified to make it non-polar by attaching long hydrocarbon chains to its surface - typically with either 8 or 18 carbon atoms in them. A polar solvent is used - for example, a mixture of water and an alcohol such as methanol. In this case, there will be a strong attraction between the polar solvent and polar molecules in the mixture being passed through the column.

There won't be as much attraction between the hydrocarbon chains attached to the silica (the stationary phase) and the polar molecules in the solution. Polar molecules in the mixture will therefore spend most of their time moving with the solvent. Non-polar compounds in the mixture will tend to form attractions with the hydrocarbon groups because of Vander Waals dispersion forces. They will also be less soluble in the solvent because of the need to break hydrogen bonds as they squeeze in between the water or methanol molecules[9].

Fig. 1.3: A typical HPLC [Water Alliance] System[10]

1.3.3 Operation of HPLC:

The mobile phase in HPLC refers to the solvent being continuously applied to the column, or stationary phase. It is stored in a reservoir and from there it is transferred to the column with the help of the action of a pump. Here, it acts as a carrier for the sample solution. A sample solution from the vials loaded into the system is injected into the mobile phase through the injector port.

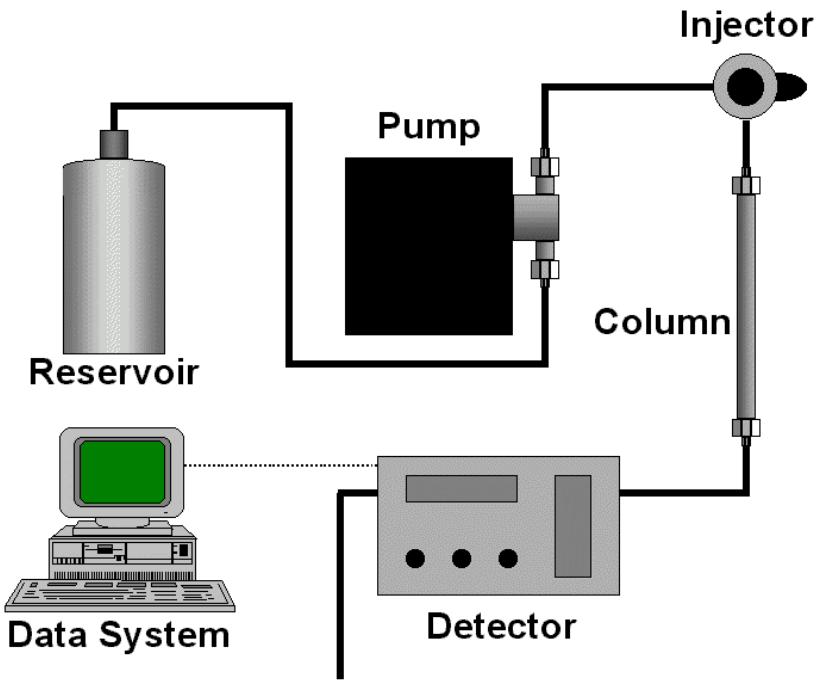


Fig. 1.4: A Flow Scheme of HPLC

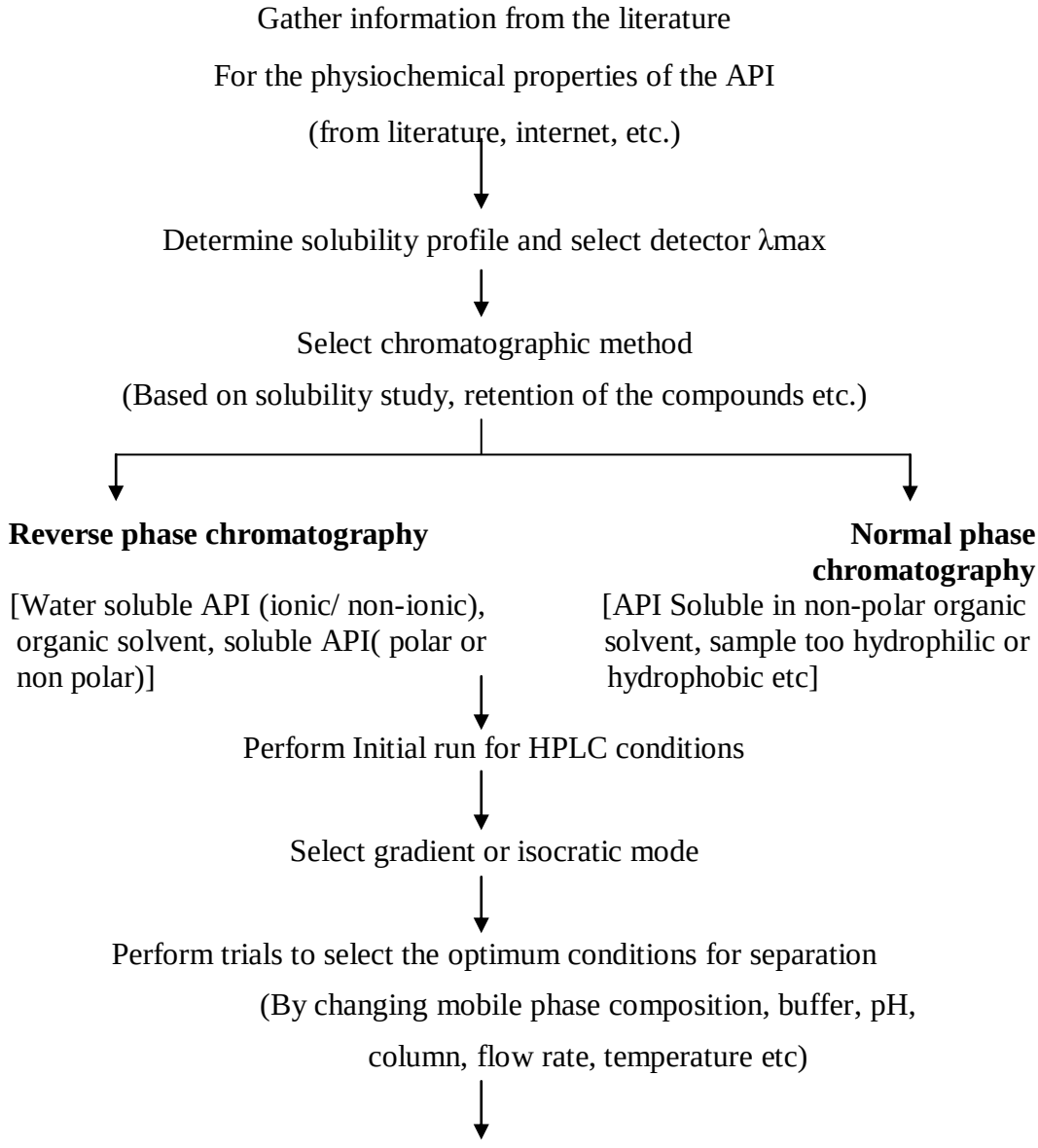
As the sample solution flows through the column with the mobile phase, the components of that solution migrate according to the non-covalent interactions of the compound with the column. The chemical interactions of the mobile phase and sample, with the column, determine the degree of migration and separation of components contained in the sample. For example, those samples which have stronger interactions with the mobile phase than with the stationary phase will elute from the column faster and thus have a shorter retention time, while the reverse is also true. The mobile phase can be altered in order to manipulate the interactions of the sample and the stationary phase. The separated components of the sample solution can be detected by the different kinds of detectors attached to the HPLC system. This would

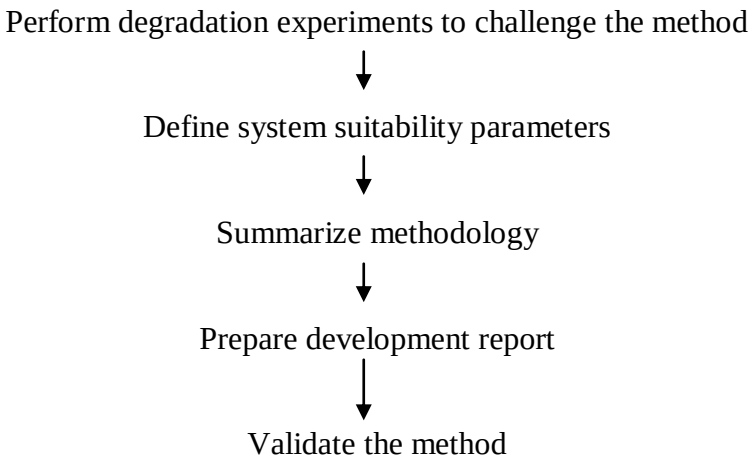
result in the generation of signals (voltages) which are represented in the form of peaks in a chromatogram. Such chromatograms are then analysed by a data system in order to obtain the amount of the analyte present.

1.4 METHOD DEVELOPMENT BY HPLC:

1.4.1 Selecting an HPLC method and initial conditions[12]

The first consideration when developing an HPLC method is to determine the solubility of the sample components. Knowing the nature of analyte will allow the most appropriate mode of HPLC to be selected. For the selection of a suitable chromatographic method for organic compounds first reversed-phase should be tried, if not successful, normal-phase should be taken into consideration.





Flow Chart 1: Method Development Flow Chart

1.5 INTRODUCTION TO STABILITY INDICATING ASSAY METHODS (SIAMs)

1.5.1 Stability indicating assay methods (SIAMs): The stability-indicating assay is a method that is employed for the analysis of stability of samples in pharmaceutical industry. With the advent of International Conference on Harmonization (ICH) guidelines, the requirement of establishment of explicitly require conduct of forced decomposition studies under a variety of conditions, like pH, light, oxidation, dry heat, etc. and separation of drug from degradation products. The method is expected to allow analysis of individual degradation products.

Stability-indicating methods according to United States-Food and Drug Administration (US-FDA) stability guideline of 1987 were defined as the 'Quantitative analytical methods that are based on the characteristic structural, chemical or biological properties of each active ingredient of a drug product and that will distinguish each active ingredient from its degradation products so that the active ingredient content can be accurately measured.' This definition in the draft guideline of 1998 reads as: 'Validated quantitative analytical methods that can detect the changes with time in the chemical, physical, or microbiological properties of the drug substance and drug product, and that are specific so that the contents of active ingredient, degradation products, and other components of interest can be accurately measured without interference[7].

1.5.2 Types of stability indicating assay method (SIAM)[15]

a) Specific stability indicating assay method: It can be defined as 'A method that is able to measure unequivocally the drug(s) in the presence of all degradation products, in the presence of excipients and additives, expected to be present in the formulation.'

b) Selective stability indicating assay method : Whereas it can be defined as 'A method that is able to measure unequivocally the drug(s) and all degradation products in the presence of excipients and additives, expected to be present in the formulation'.

1.5.3 Regulatory requirements[16]

From a regulatory perspective, forced degradation studies provide data to support the following:

- Identification of possible degradants.
- Degradation pathways and intrinsic stability of the drug molecule.
- Validation of stability indicating analytical procedures.

1.5.3.1 Issues addressed in regulatory guidance include

- Forced degradation studies are typically carried out using one batch of material.
- Forced degradation conditions are more severe than accelerated stability testing such as 50 °C; ≥75% relative humidity; in excess of ICH light conditions; high and low pH, oxidation, etc.
- Photo stability should be an integral part of forced degradation study design.
- Degradation products that do not form in accelerated or long term stability may not have to be isolated or have their structure determined.
- Mass balance should be considered.

1.5.3.2 Issues not specifically addressed in regulatory guidance:

- Exact experimental conditions for forced degradation studies (temperatures, duration, extent of degradation, etc.) are not specified.
- Experimental design is left to the applicant's discretion.

There are guidances available from the FDA as well as from private industry on regulatory requirements for IND and NDA filings[17].

1.5.4 Summary of requirements at the IND stage:

The reporting of forced degradation study conditions or results is not required in phase 1 or 2 INDs. However, preliminary studies are encouraged to facilitate the development of stability indicating methodology. It is recommended that forced degradation testing be conducted as early in the development of API and DP as possible. Studies can be conducted on the API and developmental formulations to examine for degradation by thermolysis, hydrolysis, oxidation, and photolysis to evaluate the potential chemical behavior of the active.

1.5.5 Summary of requirements for marketing application:

Completed studies of the degradation of the API and DP are required at the NDA stage, including isolation and/or characterization of significant degradation products and a full written account of the degradation studies performed[18].

1.5.5.1 Requirements at the time of registration include[19]:

- Forced degradation products should be accurately characterized and the reaction kinetics established.
- Structural elucidation of degradation products should be attempted, even if not successful, should be referenced in the NDA.
- Mass balance should be determined or at least attempted.
- Main band peak purity should be confirmed.
- Any degradants present in ICH stability samples which are greater than the identification threshold should be isolated and identified.

1.5.5.2Information from these studies should provide for filing are:

- Degradation pathways of the API alone and in DP.
- Discussion of any possible polymorphic or enantiomeric substances.

1.5.6 Stress testing guidance documents as per ICH Q1A (R2) guideline[20]:

1.5.6.1 Drug substance:

Stress testing of a drug substance can help identify the likely degradation products, which can in turn help establish the degradation pathways and the intrinsic stability of the molecule and validate the stability-indicating power of the analytical procedures used. The nature of the stress testing will depend on the individual drug substance and the type of drug product. Stress testing is likely to be carried out on a single batch of the drug substance. It should include the effect of temperatures (in 10 ^{0}C increments above that for accelerated testing), humidity (75% RH or greater) where appropriate, oxidation, and photolysis on the drug substance. Testing should evaluate the susceptibility of the drug substance to hydrolysis across a wide range of pH values when in solution or suspension. Photostability testing should be an integral part of stress testing. Examining degradation products under stress conditions is useful for establishing degradation pathways and developing and validating suitable analytical procedures. It may not be necessary, to examine for specific degradation products if previous studies have demonstrated that these products are not formed under accelerated or long-term storage conditions.

1.5.6.2 Drug product:

The design of formal stability studies for a drug product should be based on the behavior and properties of the drug substance, the results from stability studies on the drug substance, and the experience gained from clinical formulation studies. The likely changes to storage conditions and the rationale for the selection of attributes to be tested in the formal stability studies should be stated. Photostability testing should be conducted on at least one primary batch of the drug product if appropriate. Standard conditions for photostability testing are described in ICH Q1B.

Any evaluation should take into account not only the assay but also the degradation products and other appropriate attributes. Where appropriate, attention should be paid to reviewing the adequacy of the mass balance and stability and degradation performance.

1.5.7 Stress testing guidance documents as per ICH Q1B guideline[21]:

Photostability testing should consist of two parts: (1) forced-degradation testing and (2) confirmatory testing. The purpose of forced-degradation testing is to evaluate the overall photosensitivity of the material for method-development purposes and/or degradation pathway elucidation. This testing may involve the drug substance alone and/or the substance in simple solutions and suspensions to validate the analytical procedures. In these studies, the samples should be in chemically inert and transparent containers. For development and validation purposes, it is appropriate to limit exposure and end the studies if extensive decomposition occurs. The design of these experiments is left to the applicant's discretion although the exposure levels used should be justified. Under forcing conditions, decomposition products may be observed that are unlikely to be formed under the conditions used for confirmatory studies. This information may be useful in developing and validating suitable analytical methods. If in practice it has been demonstrated that they are not formed in the confirmatory studies, these degradation products need not be further examined.

1.5.7.1 Light Source used for Photostability testing is following:

- **Option 1** Any light source that is designed to produce an output similar to the D65/ID65 emission standard such as an artificial daylight fluorescent lamp combining visible and ultraviolet (UV) outputs, xenon, or metal halide lamp. D65 is the internationally recognized standard for outdoor daylight as defined in ISO 10977 (1993). ID65 is the equivalent indoor indirect daylight standard. For a light source emitting significant radiation below 320 nm, an appropriate filter(s) may be fitted to eliminate such radiation.
- **Option 2** For option 2 the same sample should be exposed to both the cool white fluorescent and near ultraviolet lamp.
    - A cool white fluorescent lamp designed to produce an output similar to that specified in ISO 10977(1993) ; and
    - A near UV fluorescent lamp having a spectral distribution from 320 nm to 400 nm with a maximum energy emission between 350 nm and 370

nm; a significant proportion of UV should be in both bands of 320 to 360 nm and 360 to 400 nm.

For confirmatory studies, samples should be exposed to light providing an overall illumination of **not less than 1.2 million lux/ hours** and an integrated near ultraviolet energy of **not less than 200 watt hours/square meter** to allow direct comparisons to be made between the drug substance and drug product, if no any degradation seen than expose the drug substance and drug product to light providing an overall illumination of **not less than 6 million lux hours,** and than also no any degradation seen than declare the drug substance and drug product to be photostable. Requirements for relevant stress conditions are following:

- Should lead to the degradation of the main compound, but not more than 5–15%.
- Should lead to a good predictability of degradation pathways (i.e., a low probability of "drastic "or "false "degradation).
- Should be conducted for no longer than three months.

	Drug Substance		**Drug Product**	
Condition	**Solid**	**Solution/ Suspension**	**Solid(Tablets, Capsules, Blends)**	**Solution (Oral Suspension)**
Acid/base	-	✓	-	×
Oxidative	×	✓	✓	✓
Photostability	✓	×	✓	✓
Thermal	✓	-	✓	✓
Thermal/humidity	✓	-	✓	-
✓ = recommended; × = optional, suggested for some compounds				

Table 1.1: General protocol for stress degradation studies (API/DP)[16]

1.5.8 Development of validated stability indicating assay methods (SIAMs):

A stability-indicating method is defined as an analytical method that accurately quantitates the active ingredients without interference from degradation products, process impurities, excipients, or other potential impurities **or** a method that accurately

quantitates significant degradants may also be considered stability-indicating. A proactive approach to developing a stability indicating HPLC method should involve forced degradation at the early stages of development with the key degradation samples used in the method development process. Forced degradation should be the first step in method development. If forced degradation studies are performed early, method development and identification of primary degradation products and unknown impurities can be run in parallel.

Step I: Critical study of the drug structure to access the likely decomposition route:

This should be the first element whenever one takes up the project on establishment of a SIAM. Much information can simply be gained from the structure, by study of the functional groups and other key components. There are defined functional group categories, like amides, esters, lactams, lactones, etc. that undergo hydrolysis, others like thiols, thioethers, etc. undergo oxidation, and compounds like olefins, aryl halo derivatives, aryl acetic acids, and those with aromatic nitro groups, N-oxides undergo photo decomposition[22].

Step II: Collection of information on physicochemical parameters:

Before method development is taken up, it is generally important to know various physicochemical parameters like pKa, log P, solubility, absorptivity and wavelength maximum of the drug in question. The knowledge of pKa is important as most of the pH- related changes in retention occur at pH of the buffer to be used in the mobile phase. The knowledge of log P for the drug and the identified degradation products provides good insight into the separation behavior likely to be obtained on a particular stationary phase.

Step III: Stress (forced decomposition) studies:

The next step in the development of SIAM is the conduct of forced decomposition studies to generate degradation products of the drug. The ICH guideline Q1A suggests the following conditions to be employed:

(i) 10 ^{0}C increments above the accelerated temperatures

(ii) Humidity where appropriate (75 % or greater)

(iii) Hydrolysis across a wide range of pH values

(iv) Oxidation

(v) Photolysis

The guideline provides no details on how hydrolytic, photolytic and oxidative studies have to be actually performed. A comprehensive document providing guidance on the practical conduct and issues related to stress testing under variety of ICH prescribed conditions has been published lately. The decision trees have been developed to help in the selection of the right type of stress condition in a minimum number of attempts. Dependent upon the results, decision is taken on whether to increase the strength of the reaction conditions.

Step IV: Preliminary separation studies on stressed samples[23]:

The stress samples obtained are subjected to preliminary analysis to study the number and types of degradation products formed under various conditions. For doing so, the simplest way is to start with a reversed-phase octadecyl column, preferably a new or the one in a healthy condition. It should be preferred to use water-methanol or water-acetonitrile as the mobile phase in an initial stage.

Step V: Final method development and optimization[24]:

To separate close or co-eluting peaks, the method is optimized, by changing the mobile phase ratio, pH, gradient, flow rate, temperature, solvent type, and the column and its type.

Step VI: Identification and characterization of degradation products and preparation of standards:

Before moving to the validation of a SIAM, it is necessary to identify the drug degradation products and arrange for their standards. These are required to establish specificity/selectivity of the method. The work on this aspect can even be initiated once an idea on the nature and number of degradation products formed under different degradation conditions is obtained from preliminary separation studies. Peak purity of the active substance is checked (by photo-diode array detector) to verify that the method is selective, and a single component peak is quantified.

Step VI: Validation of stability indicating assay methods[25]:

Validation of analytical methods, in general, has been extensively covered in the ICH guidelines Q2A and Q2B, in the FDA guidance and by USP. There are several other

reports in literature, which have reviewed the concept, either in general or specifically the validation of spectroscopic, non-chromatographic and chromatographic methods. Overall, there are two stages in the validation of a stability indicating assay method.

1) First stage is early in the development cycle when drug substance is subjected to forced decomposition studies and the SIAM is established based on the knowledge of drug degradation behavior. The main focus of validation at this stage is on establishment of specificity/selectivity, followed by other parameters like accuracy, precision, linearity, range, robustness, etc. The limits of detection and quantitation are also determined finds application in the analysis; of stability samples of bulk drug for determination of its retest or expiry period.
2) In the second stage, when the SIAM so developed is extended to formulations or other matrices, the emphasis gets limited to just prove the pertinence of the established validation parameters in the presence of excipients or other formulation constituents.

1.6 VALIDATION[25-29]

Validation is defined as follows by different agencies:

Food and Drug Administration (FDA): Establishing documentation evidence, which provides a high degree of assurance that specific process, will consistently produce a product meeting its predetermined specification and quality attributes.

World Health Organization (WHO): Action of providing that any procedure, process, equipment, material, activity, or system actually leads to the expected results.

European Committee (EC): Action of providing in accordance with the principles of good manufacturing practice, that any procedure, process, equipment material, activity or system actually lead to the expected results. In brief **validation** is a key process for effective quality assurance.

1.6.1 Objective of validation

The primary objective of validation is to form a basis for written procedures for production and process control which are designed to assure that the drug products

have the identity, strength, quality and purity they purport or are represented to process.

- Quality, safety and efficacy must be designed and built into the products.
- Each step of the manufacturing process must be controlled to maximize the probability that the finished product meets all quality and design specifications.

1.6.2 Analytical method validation

Analytical monitoring of a pharmaceutical product or of specific ingredients within the product is necessary to ensure its safety & efficacy throughout all phases of its shelf life.

Analytical validation is the corner stone of process validation without a proven measurement system it is impossible to confirm whether the manufacturing process has done what it purports to do. All new methods developed are validated.

Steps followed for validation procedures are:

a) Proposed protocols or parameters for validations are established.
b) Experimental studies are conducted.
c) Analytical results are evaluated
d) Statistical evaluation is carried out.
e) Report is prepared documenting all the results.

Analytical test method validation is completed to ensure that analytical methodology is accurate, specific, reproducible and robust over the specified range that an analyte will be analyzed. Method validation provides an assurance of reliability during normal use, and sometime referred to as "the process of providing documented evidence that the method does what it is intended to do."

Types of analytical procedures to be validated

- Identification tests
- Quantitative tests for impurities
- Limit tests for the control of impurities
- Quantitative tests of the active moiety in samples of drug substances or drug products or other selected components in the drug products

The ICH documents give guidance on the necessity for revalidation in the following circumstances:

- Changes in the synthesis of the drug substances
- Changes in the composition of the drug product
- Changes in the analytical procedures

1.6.2.1 Specificity

An investigation of specificity should be conducted during the validation of identification tests, the determination of impurities, and the assay. The procedures used to demonstrate specificity will depend on the intended objective of the analytical procedure. It is not always possible to demonstrate that an analytical procedure is specific for a particular analyte (complete discrimination).

Acceptance criteria:- Non interference

1.6.2.2 Linearity

Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods.

The correlation coefficient, y-intercept, slope of the regression line, and residual sum of squares should be submitted. A plot of the data should be included. In addition, an analysis of the deviation of the actual data points from the regression line may also be helpful for evaluating linearity. For the establishment of linearity, a minimum of five concentrations is recommended.

Acceptance criteria :- Correlation coefficient (r2) > 0.999

1.6.2.3 Range

The specified range is normally derived from linearity studies and depends on the intended application of the procedure. It is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy, and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure.

Acceptance criteria:- 80-120%

1.6.2.4. Accuracy

Accuracy should be established across the specified range of the analytical procedure. It may be inferred once precision, linearity, and specificity has been established. Accuracy should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range (e.g., 3 concentrations/ 3 replicates each of the total analytical procedure). Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals.

Acceptance criteria:- Recovery 98-102% (individual) with 80, 100, 120 % spiked sample.

1.6.2.5 Precision

Validation of tests for assay and for quantitative determination of impurities includes an investigation of precision.

a) Repeatability

Repeatability should be assessed using:

- A minimum of 9 determinations covering the specified range for the procedure (e.g., 3 concentrations/3 replicates each); or
- A minimum of 6 determinations at 100 percent of the test concentration.

Acceptance criteria for repeatability: - R.S.D. < 2 %

b) Intermediate precision

The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used. The applicant should establish the effects of random events on the precision of the analytical procedure.

Typical variations to be studied include days, analysts, equipment, etc. It is not necessary to study these effects individually. The use of an experimental design (matrix) is encouraged.

Acceptance criteria for repeatability: - R.S.D. < 2 %

c) Reproducibility

Reproducibility is assessed by means of an interlaboratory trial. Reproducibility should be considered in case of the standardization of an analytical procedure, for instance, for inclusion of procedures in pharmacopoeias. These data are not part of the marketing authorization dossier.

d) Recommended data

The standard deviation, relative standard deviation (coefficient of variation), and confidence interval should be reported for each type of precision investigated.

1.6.2.6 Limit of detection

Several approaches for determining the detection limit are possible, depending on whether the procedure is non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

a) Based on visual evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods. The detection limit is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

b) Based on signal-to-noise

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

c) Based on the standard deviation of the response and the slope

The limit of detection (LOD) may be expressed as:

$$L.O.D. = 3.3(SD/S)$$

where, SD = Standard deviation of the response

S = Slope of the calibration

The slope S may be estimated from the calibration curve of the analyte.

1.6.2.7 Limit of quantitation

Several approaches for determining the quantitation limit are possible, depending on whether the procedure is non-instrumental or instrumental. Some of the approaches are as follows:

a) Based on visual evaluation

Visual evaluation may be used for non-instrumental methods, but may also be used with instrumental methods. The quantitation limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

b) Based on signal-to-noise

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

c) Based on the standard deviation of the response and the slope

The limit of quantitation (LOQ) may be expressed as:

$$L.O.Q. = 10(SD/S)$$

where, SD = Standard deviation of the response

S = Slope of the calibration

The slope S may be estimated from the calibration curve of the analyte.

1.6.2.8 Robustness

The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in the parameters.

If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure.

1.6.2.9 System suitability testing

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated.

CHAPTER 2

REVIEW OF LITERATURE

Patil S. et al. have reported a validated LC–MS/MS method for quantification of agomelatine in human plasma and its application in a pharmacokinetic study. An analytical method based on liquid–liquid extraction has been developed and validated for analysis of agomelatine in human plasma. Fluoxetine was used as an internal standard for agomelatine. A Betasil C_{18} (4.0*100mm, 5µm) column provided chromatographic separation of analytes followed by detection with mass spectrometry. The method involved simple isocratic chromatographic conditions and mass spectrometric detection in the positive ionization mode using an API-4000 system. The proposed method validated with linear range of 0.050–8.000 ng/ml for agomelatine. The intra-run and inter-run precision values were within 12.12% and 9.01%, respectively, for agomelatine at the lower limit of quantification level. The overall recovery for agomelatine and fluoxetine was 67.10% and 72.96%, respectively. [30]

Zupancic M. et al. have reported preliminary review of agomelatin. Agomelatine is a new potent antidepressant which acting as an agonist of melatonin receptors and an antagonist of the serotonin 5-HT_2C receptor subtype. It is in late-phase trials for the treatment of major depressive disorder (MDD). The results of the study reveals that symptoms of depression significantly improved with agomelatine compared with placebo in large placebo-controlled trials, and agomelatine appears to be as efficacious in treating MDD as other antidepressants but with fewer adverse effects. Agomelatine appears to improve sleep quality and ease of falling asleep, as measured subjectively in depressed patients. Polysomnographic studies have shown that agomelatine decreases sleep latency, decreases wake after sleep onset (WASO), and improves sleep stability as measured by changes in the cyclic alternating pattern. Agomelatine is generally well tolerated in patients with MDD; in clinical trials, adverse events were generally mild to moderate in nature, with an overall frequency close to that of placebo. [31]

Rao P.V. et al have reported clinical and pharmacological review on novel melatonergic antidepressant Agomelatine. Agomelatine is a new agent with a unique pharmacological outline, as it is the first melatonergic antidepressant. It has potential role in the treatment of patients with major depressive disorder (MDD). Agomelatine (trade names Valdoxan, Melitor, Thymanax) is a chemical compound that is structurally closely related to melatonin. Agomelatine has a new pharmacological mechanism of action, which combines melatonin MT_1 and MT_2 agonist properties with a serotonin 5-HT_2C antagonist effect. Agomelatine was rapidly and well (=80%) absorbed after oral administration. Because of its action upon the melatonin receptors, agomelatine shows a marked improvement in sleep quality. [32]

Pjrek E. et al. have reported effect of agomelatin in seasonal affective disorders. The aim of the study was to investigate the efficacy and tolerability of agomelatine in the treatment of seasonal affective disorder (SAD). Thirty-seven acutely depressed SAD patients were included in an open study with agomelatine (25 mg/day in the evening) over 14 weeks. Efficacy assessments included the Structured Interview Guide for the Hamilton Depression Rating Scale (SAD version; SIGH-SAD), the Clinical Global Impression of Severity (CGI-S) and Improvement (CGI-I), the Circscreen, a self-rating scale for the assessment of sleep and circadian rhythm disorders, and the Hypomania Scale. The results of this study suggest that seasonal depression may be effectively and safely treated with agomelatine. [33]

CHAPTER 3

DRUG PROFILE

3.1 DRUG PROFILE [32, 34-38]

Agomelatine is a new agent with a unique pharmacological outline, as it is the first melatonergic antidepressant. It has potential role in the treatment of patients with major depressive disorder (MDD). Agomelatine (trade names Valdoxan, Melitor, Thymanax) is a chemical compound that is structurally closely related to melatonin. Agomelatine has a new pharmacological mechanism of action, which combines melatonin MT_1 and MT_2 agonist properties with a serotonin 5-HT_2C antagonist effect..

3.1.1 Physical and chemical properties[32]:

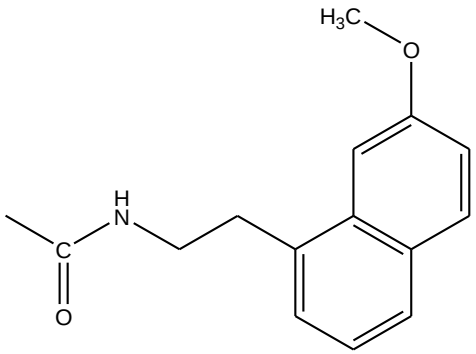
Name of drug	Agomelatine
Structure	
Category	Antidepressant
Chemical Name (IUPAC)	N-[2-(7-methoxynaphthalen-1-yl) ethyl]acetamide
Molecular formula	$C_{15}H_{17}NO_2$
Molecular weight	243.301
Occurrence	As a white or off white crystalline powder
Solubility	Insoluble in water and soluble in all organic solvents (30mg/ml).
Melting Point	108^oC
Storage	Store in a cool dry place. Protect from sunlight.

Table-3.1: Drug profile of Agomelatine

3.2 Pharmacology [32,34-38]

3.2.1 Mechanism of action:

Agomelatine is a potent agonist of melatonin (MT_1 and MT_2) receptors with 5-HT_2C antagonist properties. It is also a 5-HT_2B antagonist. Agomelatine does not interact with adenosine, adrenergic, dopamine, GABA, muscarinic, nicotinic, histamine, excitatory amino acid, benzodiazepine and sigma receptors, nor with sodium, potassium or calcium channels. Through its 5-HT_2C antagonist effect, agomelatine increases dopamine and noradrenaline release specifically in the prefrontal cortex.

3.2.2 Pharmacodynamics

3.2.2.1 Primary pharmacodynamics:

Agomelatine is a melatonin agonist with high affinity binding to human melatonine MT_1 and MT_2 receptors. Agomelatine is also a serotonin antagonist at the 5-HT_2C receptor from man and several animal species, although with low affinity. Two of the three main metabolites of agomelatine showed some pharmacological activity at the melatonin receptors, while a third metabolite (dihydrodiolagomelatine, DHDP) was not pharmacologically active at either receptor families.

3.2.2.2 Secondary pharmacodynamics:

Agomelatine showed chronobiotic activity related to the melatonin activity, and anxiolytic effects while no indication of antipsychotic properties was seen.

3.2.3 Pharmacokinetics:

Bioavailability of Agomelatin is 78% and half life is 2.3 hours.

3.2.3.1 Absorption-Bioavailability

Agomelatine was rapidly and well (≥80%) absorbed after oral administration. Absolute bioavailability is low (< 5% at the therapeutic oral dose) and the interindividual variability is substantial. *In vitro* intestinal transport of agomelatine across a Caco-2 cell monolayer was high through passive diffusion and corresponded to a predicted *in vivo* human rapid and total absorption of the compound. *In vivo*, as

reflected by the urinary recovery of radioactivity following oral administration of [$_3$H]-agomelatine and [$_{14}$C]-agomelatine gastrointestinal absorption was at least 81 ± 4.2% of the dose, and rapid (Tmax < 1h). The bioavailability is increased in women compared to men. The bioavailability is increased by intake of oral contraceptives and reduced by smoking.

Food intake (standard meal or high fat meal) did not modify the extent of bioavailability of agomelatine. Therefore agomelatine can be administered with or without meals. The variability is increased with high fat food. The peak plasma concentration is reached within 1 to 2 hours. In the therapeutic dose-range, agomelatine systemic exposure increases proportionally with dose. At higher doses, a saturation of the first pass effect occurs.

3.2.3.2 Tissue distribution and protein binding

Steady-state volume of distribution was determined as about 35L after i.v. administration of agomelatine and was dose independent. Agomelatine was bound to plasma proteins at 95% mainly to serum albumin (about 35%) and alpha1-acid glycoprotein (about 36%).

3.2.3.3 Metabolism and excretion

The main routes of metabolism in rat, monkey and man were as 3-hydroxylation, 7-desmethylation and oxidation of the naphtyl moiety at position 7, leading to the main metabolites 3HP, 7DP, and DHDP. The metabolites of agomelatine were conjugated and excreted via urine and faeces, and only low levels of unchanged agomelatine were excreted.

3.2.4 Clinical efficacy

The efficacy and safety of agomelatine in major depressive disorder were studied in a clinical development programme in which agomelatine was administered to more than 2400 patients and 400 healthy volunteers in 25 countries in Europe, Africa, South America, Australia and North America.

3.2.4.1 Adverse reactions

Adverse reactions were usually mild or moderate and occurred within the first two weeks of treatment. The most common adverse reactions were dizziness and nausea. These adverse reactions were usually transient and did not generally lead to cessation of therapy.

CHAPTER 4

RESEARCH ENVISAGED

4.1 Rationale of work

Literature study revealed that only one LC/MS/MS method is available for estimation of Agomelatine in blood plasma. There is no single analytical method is reported for the estimation of Agomelatine in bulk drug or pharmaceutical marketed formulation as well as no analytical method is available for stability indicating assay.

So, it was endeavoured to develop and validate simple, accurate, precise, specific, selective, rugged UV-Visible spectrophotometric as well as stability indicating RP-HPLC assay method for estimation of Agomelatin in bulk drug and pharmaceutical dosage form.

4.2 Objective:

Quantitative analysis of any drug is an important tool in an industry. It is important to determine that the raw material, intermediate products as well as final products meet its specifications and are of required quality. The numbers of drugs and drug formulation introduced into market has been increasing at an alarming rate. These drugs or formulations may be either new entities or partial structural modification of existing ones or novel dosage forms.

Stability testing of an active substance or finished product provide evidence on how the quality of a drug substance or drug product varies with time influenced by a variety of environmental factors such as pH, temperature, humidity and light. Knowledge from stability studies enables understanding the long-term effects of the environment on the drugs. Stability testing provides information about degradation mechanisms, potential degradation products, possible degradation pathways of drug as well as interaction between the drug and the excipients in drug product.

Although, the concept of stress testing is not new to the pharmaceutical industry, the procedure was not clearly defined until the International Conference on Harmonization (ICH) provided a definition in its guidance on stability. The ICH guideline indicates that stress testing is designed to help "determine the intrinsic stability of the molecule by establishing degradation pathways in order to identify the likely degradation products and to validate the stability indicating power of the analytical procedures used"[12, 14]

4.3 Plan of work

4.3.1 Identification of drug

- Melting point determination by DSC
- Identification by U.V. Spectroscopy
- Identification by FT-IR spectroscopy
- Identification by Mass spectroscopy

4.3.2 UV Spectrophotometric method development

- Study of physiochemical parameters.
- Check the solubility of sample
- Selection of solvent
- Selection of detection wavelength

4.3.3 RP-HPLC method development

- Study of physiochemical parameters.
- Check the solubility of sample.
- Need for special HPLC procedure, sample pre-treatment.
- Choose the appropriate mobile phase.
- Choose the appropriate columns.
- Choose the appropriate diluents.
- Choose detector and detector settings.

4.3.3 Forced degradation study

- Neutral hydrolysis
- Acid degradation
- Alkali degradation
- Peroxide degradation
- Thermal degradation
- Photolytic degradation

4.3.4 Validation of developed method

- System suitability

- Specificity
- Accuracy
- Precision
- Limit of detection
- Limit of quantitation
- Linearity
- Range
- Solution stability
- Robustness

CHAPTER 5

MATERIALS & INSTRUMENTS

5.1 INSTRUMENTS:

Sr. No	Name	Model	Manufacturer/ Supplier
1	pH meter	780	Metrohm
2	Sonicator	SC 133	Flexit jour lab pvt. ltd.
3	UV-Visible Spectrophotometer	1800	Shimadzu
4	HPLC system	LC 2010 C with auto sampler	Shimadzu
5	HPLC system	1200 series with auto injector	Agilent
6	Columns	Zorbax XBD C_8 & C_{18}	Agilent
7	Analytical balance	XP205	Mettler Toledo
8	Analytical micro balances	ME5	Sartorius
9	FTIR	FTIR-8400	Shimadzu
10	Photo stability chamber	SUNTEST XLS+	Atlas MTT Gmbh
11	Mass Spectrophotometer	API-3200	APC-IEX (Canada)
12	DSC	Pyris-1	Perkin Elmer

Table-5.1: List of Apparatus / Instruments used in Experimental work

5.2 CHEMICALS:

Sr.No.	Name	Specification	Manufacturer / Supplier
1	Milli-Q water	-	Barnstead
2	Acetonitrile	HPLC Grade	Spectrochem Pvt. Ltd., Mumbai
3	Methanol	HPLC Grade	Spectrochem Pvt. Ltd., Mumbai
4	Triethylamine	HPLC Grade	Spectrochem Pvt. Ltd., Mumbai.
5	Ortho-phosphoric acid	HPLC Grade	Spectrochem Pvt. Ltd., Mumbai.

Table-5.2: List of Chemicals used in Experimental work

5.3 API AND FORMULATIONS:

Drugs and Ingredients	Manufacturers
Agomelatine working standard	Zydus Cadila Healthcare Ltd.
Agomelatine sample (In house prepared tablets 25mg)	Zydus Cadila Healthcare Ltd.
Agomelatine tablet placebo	Zydus Cadila Healthcare Ltd.

Table-5.3: Drugs and Formulation

CHAPTER 6

IDENTIFICATION OF DRUG

6.1 CHARACTERISATION AND IDENTIFICATION AGOMELATINE

6.1.1Solubility Study

Solubility of Agomelatine working standard was carried out in different solvents. From this study following results were obtained.

Solvent	Observed	Reported[32]
Water	Insoluble	Insoluble
Methanol	Soluble	Soluble
Acetonitrile	Soluble	Soluble

Table 6.1: Results for solubility studies

6.1.2 Melting point determination (API)

Melting range of Agomealtine was found to be 107°C to 109°C and the melting point was found to be 108°C.

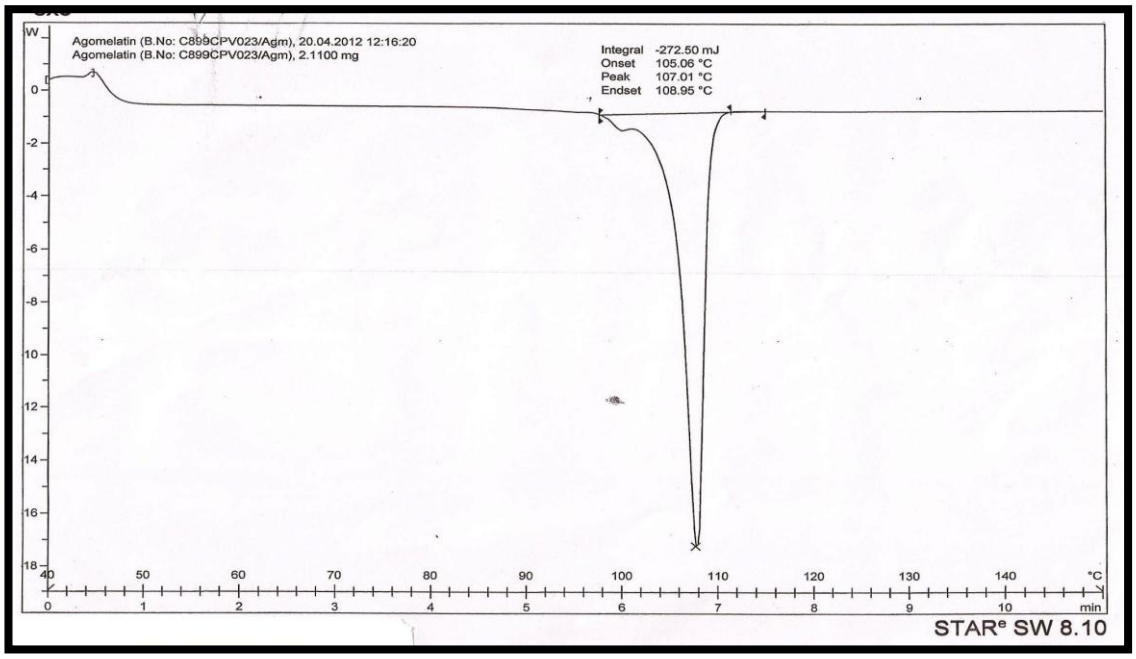


Fig. 6.1: DSC thermogram of Agomelatine

Drug	Reported[32]	Observed
Agomelatine	108°C	108°C

Table 6.2: Melting point determination of Agomelatine by DSC

6.1.3 UV Spectra of Agomelatine (API)

UV Spectra of 5µg/ml of Agomelatine in methanol was taken.

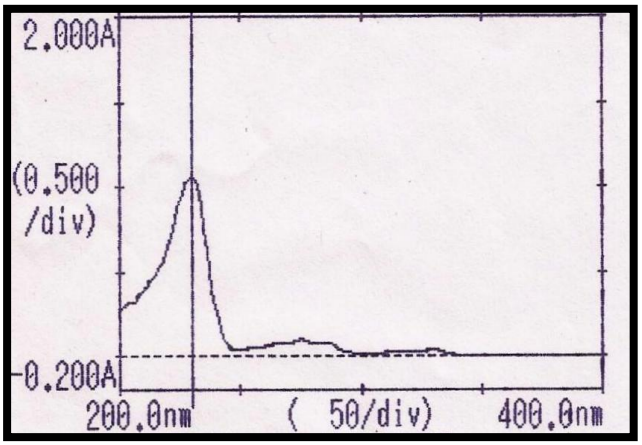


Figure 6.2: UV Spectra of Agomelatine in methanol (5µg/ml)

Drug	Observed	Reported[32]
Agomelatine	230nm	230nm

Table 6.3: Peak (Wavelength Maxima) selection of Agomelatine

6.1.4 FT- IR Spectra of Agomelatine (API)

FT-IR Spectra of Agomelatine workin standard was taken with FT-IR instrument and identification of characteristic peaks was done.

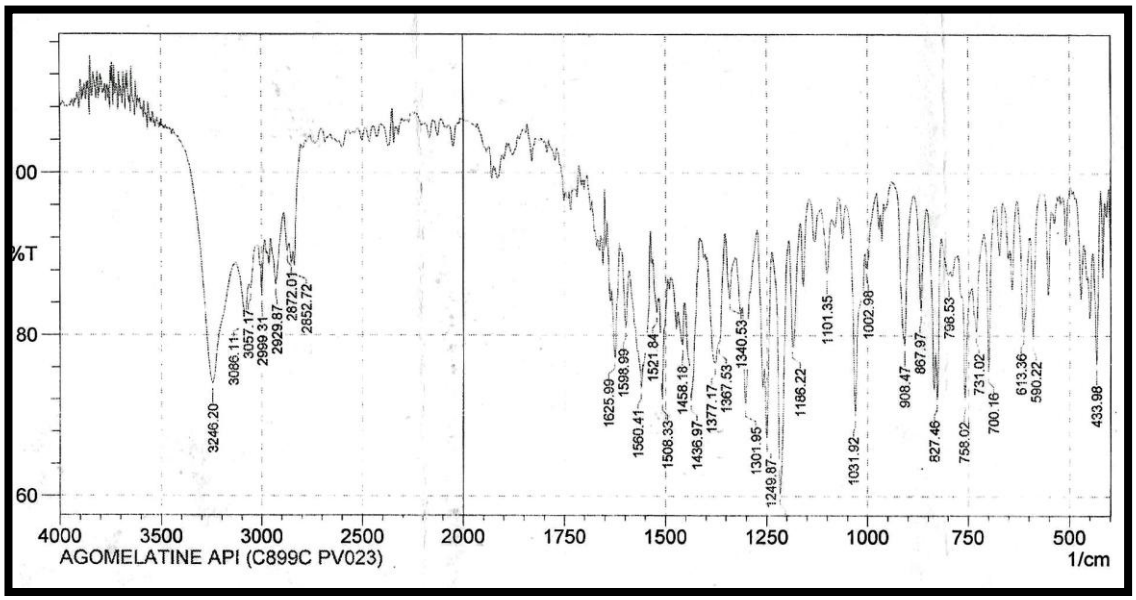


Figure 6.3: FT-IR Spectra of Agomelatine

Sr.No	Wavenumber (cm^{-1})	Functional group assignment
1	3246	N-H stretching.
2	3086	Aromatic –CH stretching
3	2929	Aliphatic –CH stretching
4	1625	–C=O stretching of acetamide
5	1508	C-O-C stretching
6	750	Aromatic –CH out-of-plane stretching

Table 6.4: FT-IR Specifications of Agomelatine

6.1.5 Mass spectra of Agomelatine (API)

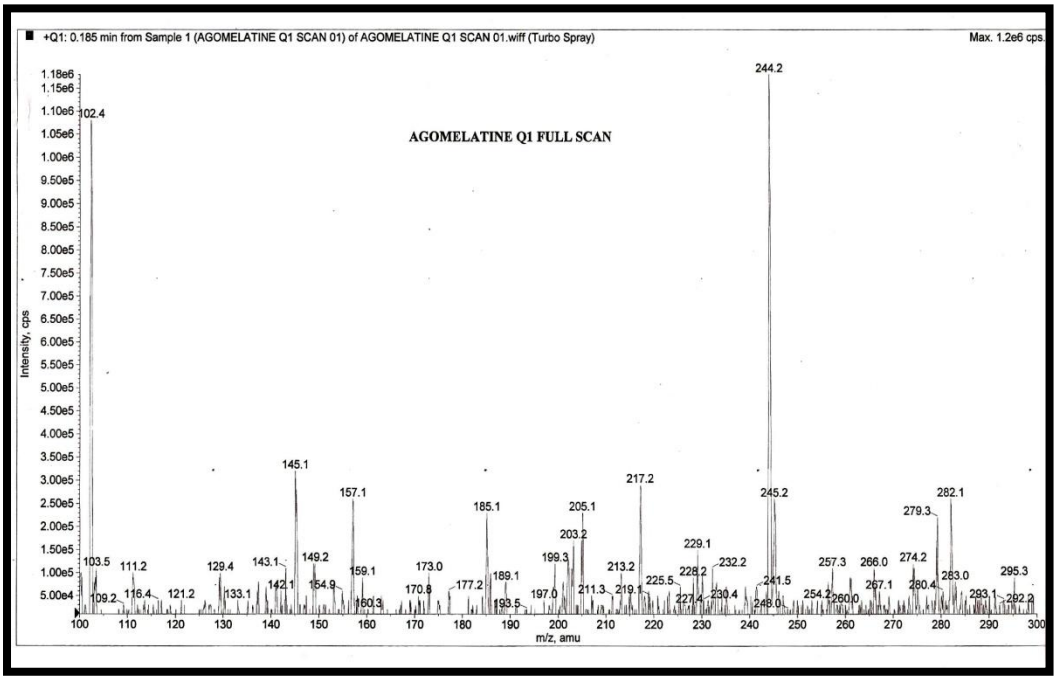


Figure 6.3: Mass Spectra of Agomelatine (API)

A mass spectrum was taken using Mass spectrophotometer. The $M+1^{+}$ molecular ion peak. Was observed at m/z (Mass to charge ratio) Hence it was confirmed that the molecule has 243.30 molecular weight. Which is exact of Agomelatine.

CHAPTER 7

EXPERIMENTAL WORK

7.1 DEVELOPMENT AND VALIDATION OF UV SPECTROPHOTOMETRIC METHOD FOR THE ESTIMATION OF AGOMELATINE IN BULK AND PHARMACEUTICAL FORMULATION

7.1.1 METHOD DEVELOPMENT

7.1.1.1 Selection of solvent

Mixture of Water and Acetonitrile in the ratio of (50:50) was selected as solvent.

7.1.2.1 Preparation of working standard stock solution

25mg of Agomelatine working standard was weighed and taken in to 100mL volumetric flask. 30 mL of solvent was added and it was kept for ultrasonication for about 5 minutes with intermittent shaking. Volume was made up to the mark with the solvents(250µg/mL). Further take 5mL of this solution was taken in 50mL volumetric flak and volume was made up to the mark with the solvent to give a final solution containing 25µg/mL of Agomelatine.

7.1.2.2 Preparation of sample stock solution:

20 tablets were weighed and finely crushed. From this 157 mg of powder equivalent to 25 mg of Agomelatine was taken into 100 mL volumetric flask. 30 mL of solvents was added and it was kept for ultrasonication for about 15 min with intermittent shaking. Volume was made up to the mark with the solvent. Solution was filtered through 0.45µ PVDF filter. From that 5mL of the filtrate was further diluted to 50mL with the solvent. (25µg/mL Agomelatine).

7.1.2.3 Blank preparation :

132 mg of placebo was taken in 100 mL volumetric flask. 30mL of solvents was added and it was kept for ultrasonication for about 15 min with intermittent shaking. Volume was made up to the mark with the solvent. Solution was filtered through 0.45µ PVDF filter. From that 5mL of the filtrate was further diluted to 50mL with the solvent.

7.1.3 Method validation parameters

7.1.3.1 Linearity and range

Aliquots of 1mL, 2mL, 3mL, 4mL, 5mL, 6mL, 7mL and 8mL from working standard stoke solution(5.1.2.1) of Agomelatine was transferred to different volumetric flask of 50mLcapacity. Volume was adjusted up to mark with the solvent to obtain concentration of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 µg/mL of Agomelatine .
Absorbance was noted at 230nm.

Acceptance criteria: Correlation coefficient should not be less than 0.999.

7.1.3.2 Accuracy (Recovery) study:

Procedure (Standard addition method)

The accuracy of the method was determined by calculating percentage recovery of Agomelatine from placebo. Recovery studies were carried out by applying the spiking method in which known amount of Agomelatine API corresponding to 50, 100 and 150% of label claim had been added to the 132mg of placebo. At each level of the amount three determinations were performed and the results obtained were compared.

Acceptance criteria: % Recovery should be in the range of 98-102%.

7.1.3.3 Precision:

a) Method precision (Repeatability)

Procedure

Method precision was done by repeatedly analyzing concentration equivalent to the assay concentration. 5mL from the working standard sock solution was taken in 50mL volumetric flask and made up to the mark with the solvent (2.5µg/mL of Agomelatine).Six determinations were done of the same. % RSD was calculated for the results obtained and recorded.

b) Intraday precision:

Procedure

Intraday precision was done by analyzing 3 different concentration from linearity range prepared under same conditions. Here 1.5, 2.5 and 3.5 µg/mL concentration were taken and analysis was done at 3 different time intervals within the day.

c) Interday precision:

Procedure

Interday precision was done by analyzing 3 different concentration from linearity range prepared under same conditions. Here 1.5, 2.5 and 3.5 µg/mL concentration were taken and analysis was done on 3 different days.

Acceptance criteria: % Relative standard deviation should be less than 2.

7.1.3.4 Solution stability:

Procedure

Absorbance of working standard stock solution was taken at 230nm at different time intervals for 36 hrs to observe difference in absorbance relative to freshly prepared stock solution.

Acceptance criteria: The difference in the absorbance should not be exceed than 2%.

7.1.3.5 LOD and LOQ

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated using following formulae: LOD= 3.3(SD)/S and LOQ= 10 (SD)/S, where SD= Standard deviation of y-intercepts of regression lines and S= average of the slope of the calibration curve.

7.1.3.6 Specificity:

Procedure

- Placebo solution was scanned as per the method.

Acceptance criteria: The difference in the absorbance should not be more than 2%.

7.1.4 CALCULATION FOR ASSAY:

Procedure:

From the sample stock solution (25µg/mL) 5mL was transferred in 50mL volumetric flask and volume was made up to the mark with the solvent. Absorbance was measured at sampling wavelength of Agomelatine.

$$\%Assay = \frac{AT}{AS} \times \frac{WS}{100} \times \frac{5}{50} \times \frac{100}{WT} \times \frac{50}{5} \times \frac{AW}{LC} \times P$$

Where,

AS = Average absorbance of Agomelatine in standard preparation

AT = Average absorbance of Agomelatine in sample preparation

WS = Weight of Agomelatine reference / working standard in mg

WT = Weight of sample in mg

AW= average weight of tablet in mg

LC= Label claim of Agomelatinein tablet in mg (25 mg)

P= Potency of Agomelatineworking/reference standard (% as such) (99.00%)

Acceptance criteria: % Assay should be in the range of 98-102%.

7.2 DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC ASSAY METHOD FOR ESTIMATION OF AGOMELATINE IN BULK DRUG AND PHARMACEUTICAL FORMULATION

7.2.1 Selection of Analytical Parameters

7.2.1.1 Selection of the detection wavelength

Absorption maxima (λmax) is the point at which a drug shows its maximum absorbance. In the quantification of drug, it is very necessary to have the peak height (response) with sufficient absorbance. So, in order to achieve proper peak response, the UV Spectra of the drug containing 2.5µg/mL concentration was prepared in the diluent (water: ACN, 62:32) and the spectrum was scanned in the range of 190-400 nm to find the maximum absorbance of the drug. The absorption maxima was found to be at 230.00 nm which was selected as the analytical wavelength.

7.2.1.2 Selection of column

The Agilent ZORBAX Eclipse XDB columns – C_{18}, C_8, Phenyl and CN – provide four bonded phase choices for method development optimization. These columns provide good peak shape over a wide pH range (2-9) for additional method development flexibility with one family of columns. Eclipse XDB columns can be used for method development at low pH (2-3) and the same column can be used for method development in the mid pH (6-8) region. In the mid pH region residual silanols are more active and tailing interactions are more likely. To overcome these interactions, Eclipse XDB columns are extra Densely Bonded and double endcapped through a proprietary process to cover as many active silanols as possible. The result is superior peak shape of basic compounds from pH 2-9.

Because of such excellent features it was thought to initiate the method development work with this column.

The column here used was ZORBAX Eclipse XDB-C_{18} column 150 × 4.6 mm internal diameter and particle size of 5 µ.

Technical Specifications of Zorbax XDB C_{18}	
Phase:	C_{18}, ODS
Particle diameter:	5.0µm
Pore diameter:	80 Angstrom
Surface area:	$180m^2/g$
Carbon load:	10%
Temperature limit:	60ºC
pH range:	2.0-9.0
End capped:	Double
Column length:	150mm
Column diameter:	4.6mm

Table 7.1: Technical Specification of Zorbax XDB C_{18}

7.2.1.3 Optimization of mobile phase and chromatographic condition

Mobile phase optimization was performed with many trials. The main purpose of the optimization was to achieve proper peak shape with sufficient height, theoretical plates, resolution and purity.

7.2.2 Preparation of Standard Solution

7.2.2.1 Standard stock solution

Accurately 25mg of Agomelatine was weighed and taken into 100 mL of volumetric flask. The compound was first dissolved with methanol. The volume was then made with the same (250µg/mL).

7.2.2.3 Sample stock solution

20 tablets were accurately weighed and crushed, equivalent to 25mg of Agomelatine tablet powder was transferred in to 100 mL volumetric flask. Around 50 mL of methanol was added and the mixture was sonicated for 15 min. The volume was then made up to mark with methanol to obtain a concentration of 250 µg/mL. This solution was filtered using 0.45µ PVDF filter.

7.3 FORCED DEGRADATION STUDIES:

Forced degradation studies of Agomelatine were carried out under conditions of Neutral-acid-alkali hydrolysis, dry heat, oxidation and photolysis. 10 µl of below prepared solutions were injected into HPLC system and analyzed under the optimised chromatographic condition described earlier.

Selection of diluent

Here the dilutions were done with the water:ACN in the ratio of 62:38. The ratio here taken was same as for the optimised mobile phase.

7.3.1 Preparation of samples without degradation

7.3.1.1 Standard solution of Agomelatine

20 mg of the drug was weighed and transferred to a 50mL volumetric flask. 25mL of methanol was added to dissolve and volume made up to 50 mL with methanol (400 µg /mL). Aliquot of 3 mL was then transferred to a 50 mL volumetric flask and volume made up to 50 mL with the diluent to obtain a solution of 24 µg /mL.

7.3.1.2 Placebo solution of Agomelatine

Placebo powder (105.6 mg) equivalent to 20 mg of Agomelatine was weighed and transferred to a 50 mL volumetric flask. 25 mL of methanol was added. The solution was sonicated for 15 min and volume made up to 50 mL with methanol. Aliquot of 3 mL was then transferred to a 50 mL volumetric flask and volume made up to 50 mL with the diluent. The solution was further filtered using 0.45µ PVDF filter.

7.3.1.3 Sample solution of Agomelatine

Tablet powder (125.6mg) equivalent to 20 mg of the drug was weighed and transferred to a 50 mL volumetric flask. 25 mL of methanol was added. The solution was sonicated for 15 min and volume made up to 25 mL with methanol. Aliquot of 3 mL was then transferred to a 50 mL volumetric flask and volume made up to 50 mL with the diluent to obtain concentration of 24 µg /mL. The solution was further filtered using 0.45µ PVDF filter.

7.3.3 Preparation for Neutral hydrolysis degradation:

7.3.3.1 Standard solution of Agomelatine

20 mg of Agomelatine was weighed and transferred to a 50 mL volumetric flask. 10 mL of water was added to prepare suspension of the Agomelatine. The suspension was heated for 8 hrs at 90°C. The suspension was cooled and volume made up to 50 mL with methanol. Aliquot of 3 mL was then transferred to a 50 mL volumetric flask and volume made up to 50 mL with the diluent to obtain concentration of 24 µg /mL.

7.3.3.2 Placebo solution of Agomelatine

Placebo powder (105.6 mg) equivalent to 20 mg of the drug was weighed and transferred to a 50 mL volumetric flask. 20 mL of water was added to prepare suspension. The suspension was heated for 8 hrs at 90°C. The suspension was cooled and volume made up to 50 mL with methanol. 3 mL of this solution was then transferred to a 50 mL volumetric flask and volume made up to 50 mL with the diluent. The solution was further filtered using 0.45µ PVDF filter.

7.3.3.3 Sample solution of Agomelatine

Tablet powder (125.6mg) equivalent to 20 mg of the drug was weighed and transferred to a 50 mL volumetric flask. 20 mL of water was added to make suspension. The suspension was heated for 8 hrs at 90°C. The solution was cooled and volume made up to 50 mL with the diluent. Aliquot of 3 mL was then transferred to a 50 mL volumetric flask and volume made up to 50 mL with the diluents to obtain a solution of 24 µg /mL. The solution was further filtered using 0.45µ PVDF filter.

7.3.3 Preparation for acid degradation:

7.3.3.1 Standard solution of Agomelatine

20 mg of the drug was weighed and transferred to a 50 mL volumetric flask. 10 mL of water was added to prepare suspension of the Agomelatine. 5 mL of 1 N HCl was added to this solution and heated for 2 hrs at 70°C. The solution was cooled, neutralised with 5 mL of 1 N NaOH and volume made up to 50 mL with methanol. 3 mL of this solution was then transferred to a 50 mL volumetric flask and volume

made up to 50 mL with the diluent to obtain a solution of 24 µg /mL. Same experiment was done by increasing degradation time period (4 hrs).

7.3.3.2 Placebo solution of Agomelatine

Placebo powder (105.6mg) equivalent to 20 mg of the drug was weighed and transferred to a 50 mL volumetric flask. 10 mL of water was added to prepare suspension. The suspension was sonicated for 15 min. 5 mL of 1 N HCl was added to this solution and heated for 2 hrs at 70°C. The solution was cooled, neutralised with 5 mL of 1 N NaOH and volume made up to 50 mL with methanol. 3 mL of this solution was then transferred to a 50 mL volumetric flask and volume made up to 50 mL with the diluent. The solution was further filtered using 0.45µ PVDF filter.

7.3.3.3 Sample solution of Agomelatine

Tablet powder (125.6mg) equivalent to 20 mg of the drug was weighed and transferred to a 50 mL volumetric flask. 10 mL of water was added to make suspension. The suspension was sonicated for 30 min. 5 mL of 1 N HCl was added to this solution and heated for 2 hrs at 70°C. The solution was cooled, neutralised with 5 mL of 1 N NaOH and volume made up to 50 mL with methanol. 3 mL of this solution was then transferred to a 50 mL volumetric flask and volume made up to 50 mL with the diluents to obtain a solution of 24 µg /mL. The solution was further filtered using 0.45µ PVDF filter. Same experiment was done by increasing degradation time period (4 hrs).

7.3.4 Preparation for alkali degradation:

7.3.4.1 Standard solution of Agomelatine

20 mg of the drug was weighed and transferred to a 50 mL volumetric flask. 10 mL of water was added to prepare suspension. 5 mL of 1 N NaOH was added to this suspension and heated for 1 hr at 70°C. The suspension was cooled, neutralised with 5 mL of 1 N HCl and volume made up to 50 mL with methanol. 3 mL of this solution was then transferred to a 50 mL volumetric flask and volume made up to 50 mL with the diluent to obtain a solution of 24 µg /mL. Same experiment was done by increasing degradation time period (2 hrs).

7.3.4.2 Placebo solution of Agomelatine

Placebo powder (105.6mg) equivalent to 20 mg of the drug was weighed and transferred to a 50mL volumetric flask. 10 mL of water was added to prepare suspension. The suspension was sonicated for 15 min. 5 mL of 1 N NaOH was added to this solution and heated for 1 hrs at 70°C. The suspension was cooled, neutralised with 5 mL of 1 N HCl and volume made up to 50 mL with methanol. 3 mL of this solution was then transferred to a 50 mL volumetric flask and volume made up to 50 mL with the diluent. The solution was further filtered using 0.45µ PVDF filter.

7.3.4.3 Sample solution of Agomelatine

Tablet powder (125.6mg) equivalent to 20 mg of the drug was weighed and transferred to a 50 mL volumetric flask. 10 mL of water was added. The solution was sonicated for 15 min. 5 mL of 1 N NaOH was added to this solution and heated for 1 hrs at 70°C. The solution was cooled, neutralised with 5 mL of 1 N HCl and volume made up to 50 mL with methanol. 3 mL of this solution was then transferred to a 50 mL volumetric flask and volume made up to 50 mL with the diluent to obtain a solution of 24 µg /mL. The solution was further filtered using 0.45µ PVDF filter. Same experiment was done by increasing degradation time period (2 hrs).

7.3.5 Preparation for peroxide degradation:

7.3.5.1 Standard solution of Agomelatine

20 mg of the drug was weighed and transferred to a 50 mL volumetric flask. 10 mL of water was added to prepare suspension. 5 mL of 30% H_2O_2 was added to this suspension and heated for 2 hrs at 80°C. The solution was cooled and volume made up to 50 mL with methanol. 3 mL of this solution was then transferred to a 50 mL volumetric flask and volume made up to 50 mL with methanol to obtain a solution of 24 µg /mL.

7.3.5.2 Placebo solution of Agomelatine

Placebo powder (105.6mg) equivalent to 20 mg of the drug was weighed and transferred to a 50 mL volumetric flask. 10 mL of water was added to prepare suspension. The suspension was sonicated for 15 min. 5 mL of 30% H_2O_2 was added to this solution and heated for 2 hrs at 80°C. The solution was cooled and volume

made up to 50 mL with methanol. 5 mL of this solution was then transferred to a 50 mL volumetric flask and volume made up to 50 mL with the diluent. The solution was further filtered using 0.45µ PVDF filter.

7.3.5.3 Sample solution of Agomelatine

Tablet powder (125.6mg) equivalent to 20 mg of the drug was weighed and transferred to a 50 mL volumetric flask. 10 mL of water was added to prepare suspension. The suspension was sonicated for 15 min. 5 mL of 30% H_2O_2 was added to this solution and heated for 2 hrs at 80°C. The solution was cooled and volume made up to 50 mL with methanol. 3 mL of this solution was then transferred to a 50 mL volumetric flask and volume made up to 50 mL with the diluent to obtain a solution of 25 µg /mL. The solution was further filtered using 0.45µ PVDF filter.

7.3.6 Preparation for thermal degradation:

6.3.6.1 Standard solution of Agomelatine

A small amount of the drug was kept in Petri dish in oven at 100°C for 48 hrs. 20 mg of the drug was weighed and transferred to a 50 mL volumetric flask. 25 mL of methanol was added to dissolve and volume made up to 50 mL with methanol 3 mL of this solution was then transferred to a 50 mL volumetric flask and volume made up to 50 mL with the diluent to obtain a solution of 24 µg /mL.

7.3.6.2 Placebo solution of Agomelatine

A small amount of the placebo powder was kept in Petri dish in oven at 100°C for 48 hrs. Placebo powder (105.6mg) equivalent to 20 mg of the drug was weighed and transferred to a 50 mL volumetric flask. 25 mL of methanol was added. The solution was sonicated for 15 min and volume made up to 50 mL with methanol. 3 mL of this solution was then transferred to a 50 mL volumetric flask and volume made up to 50 mL with the diluent. The solution was further filtered using 0.45µ PVDF filter.

7.3.6.3 Sample solution of Agomelatine

A small amount of the tablet powder was kept in Petri dish in oven at 100°C for 48 hrs. Tablet powder (125.6mg) equivalent to 20 mg of the drug was weighed and transferred to a 50 mL volumetric flask. 25 mL of methanol was added. The solution

was sonicated for 15 min and volume made up to 50 mL with methanol. 3 mL of this solution was then transferred to a 50 mL volumetric flask and volume made up to 50 mL with the diluent to obtain a solution of 24 µg /mL. The solution was further filtered using 0.45µ PVDF filter.

7.3.7 Preparation for photo degradation:

7.3.7.1 Standard solution of Agomelatine

A small amount of the drug was kept in Petri dish in SUNTEST-XLS+ for 1.2 million lux hours. 20 mg of the drug was weighed and transferred to a 50 mL volumetric flask. 25 mL of methanol was added to dissolve and volume made up to 50 mL with methanol. 3 mL of this solution was then transferred to a 50 mL volumetric flask and volume made up to 50 mL with the diluent to obtain a solution of 24 µg /mL. The solution was further filtered using 0.45µ PVDF filter.

7.3.7.2 Placebo solution of Agomelatine

A small amount of the placebo was kept in Petri dish in SUNTEST-XLS+ for 1.2 million lux hours. Placebo powder (105.6mg) equivalent to 20 mg of the drug was weighed and transferred to a 50 mL volumetric flask. 25 mL of methanol was added. The solution was sonicated for 15 min and volume made up to 50 mL with methanol. 3 mL of this solution was then transferred to a 50 mL volumetric flask and volume made up to 50 mL with the diluent. The solution was further filtered using 0.45µ PVDF filter.

7.3.7.3 Sample solution of Agomelatine

A small amount of the tablet powder was kept in Petri dish in SUNTEST-XLS+ for 1.2 million lux hours. Tablet powder (125.6mg) equivalent to 20 mg of the drug was weighed and transferred to a 50 mL volumetric flask. 25 mL of methanol was added. The solution was sonicated for 15 min and volume made up to 50 mL with methanol. 3 mL of this solution was then transferred to a 50 mL volumetric flask and volume made up to 50 mL with the diluent to obtain a solution of 24 µg /mL. The solution was further filtered using 0.45µ PVDF filter.

Percent degradation was calculated by comparing the areas of the degraded peaks in each degradation condition with the corresponding areas of the peaks of drug under non degradation condition. Summary of degradation studies of the drug is given in

$$\% \text{ Degradation} = \frac{\text{Area dropped of sample}}{\text{Area of standard}} \times \frac{\text{Standard weight}}{\text{Standard Dilution factor}} \times \frac{\text{Sample Dilution Factor}}{\text{Sample weight}} \times \text{Potency}$$

.

7.4 METHOD VALIDATION:

The method of analysis was validated as per the recommendations of ICH [26, 27] for the parameters like accuracy, linearity, precision, specificity, detection limit, quantitation limit, robustness and solution stability.

7.4.1 System Suitability: (Repeatability)

System suitability tests are an integral part of chromatographic method which is used to verify reproducibility of the chromatographic system. To ascertain its effectiveness, certain system suitability test parameters were checked by repetitively injecting the drug solution at the concentration level 25 µg/mL Agomelatineto check the reproducibility of the system.

7.4.2 Linearity:

A 25 mg of standard Agomelatine was weighed and transferred to 100 mL of volumetric flask and dissolved with methanol., The solution was sonicated for 5 minutes. The flask was shaken and volume was made up to the mark with the methanol to get solution containing 250 µg/mL of Agomelatine. From this, aliquots of 2, 3, 4, 5, 6, 7 and 8 mL were transferred to the 50 mL of volumetric flasks and the volume was made up to the mark with the diluent to get the concentration of 10, 15, 20, 25, 30, 35 and 40 µg/mL. For estimation of Agomelatine, calibration curve was plotted in the range of 10- 40 µg/mL.

Acceptance criteria: Correlation coefficient should not be less than 0.999.

7.4.3 Accuracy:

The accuracy of the method was determined by calculating percentage recovery of Agomelatine. Recovery studies were carried out by applying the spiking method in which known amount of Agomelatine API corresponding to 50, 100 and 150% of label claim had been added to placebo. At each level of the amount three determinations were performed and the results obtained were compared.

Acceptance criteria: % Recovery should be in the range of 98-102%.

7.4.4 Precision

The intraday precision was carried out by taking the chromatogram of three different concentration (10, 25, 40 µg/mL) with the repetition of 3 times in a day and interday precision was carried out by taking three different concentration (10, 25, 40 µg/mL) with the repetition of 3 times on a three different days.

Acceptance criteria: % Relative standard deviation should be less than 2.

7.4.6 LOD and LOQ:

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated using following formulae: LOD= 3.3(SD)/S and LOQ= 10 (SD)/S, where SD= Standard deviation of y-intercepts of regression lines and S= average of the slope of the calibration curve.

7.4.7 Specificity:

To carryout specificity test injection of placebo solution was injected to check whether the placebo does not interfere with the drug peak. The drug peak was also checked for its peak purity in presence of excipients.

7.4.8 Robustness:

The robustness studies were performed on the Escitalopram API with the deliberate changes in the flow rate, wavelength, mobile phase pH and change in the column temperature.

7.4.9 Solution Stability:

The standard and sample solutions were injected at different time intervals for 36 hrs to observe % difference in chromatographic responses relative to freshly prepared solutions.

Acceptance criteria:

The peak area of analyte in standard and sample solution should not differ by more than 2.0% from initial peak area for the accepted storage time.

7.5 ESTIMATION OF AGOMELATINE TABLET BY THE DEVELOPED METHOD

Total 20 tablets (Agomelatine) were weighed accurately and powdered. An amount equivalent to 25 mg of Agomelatine was taken and dissolved in 50 mL methanol in 100 ml volumetric flask. The solution was sonicated for 15 minutes and then diluted with methanol up to the mark. From this aliquot of 5 mL was taken in 50 mL volumetric flask and volume made up to the mark with the diluent. The solution was filtered by using 0.45 µ PVDF filter. The filtered solution was injected and the chromatogram was recorded

Formula for Agomelatine:

$$\%Assay = \frac{AT}{AS} \times \frac{WS}{100} \times \frac{5}{50} \times \frac{100}{WT} \times \frac{50}{5} \times \frac{AW}{LC} \times P$$

Where,

AS = Average area of Agomelatine peak in standard preparation

AT = Average area of Agomelatine peak in sample preparation

WS = Weight of Agomelatine reference / working standard in mg

WT = Weight of sample in mg

AW= average weight of tablet in mg

LC= Label claim of Agomelatinein tablet in mg (25 mg)

P= Potency of Agomelatineworking/reference standard (% as such)

Acceptance criteria: % Assay should be in the range of 98-102%.

CHAPTER 9

SUMMARY & CONCLUSION

Summary

UV Spectrophotometric method that involves estimation of Agomelatine at wavelength 230 nm (λmax of AGO) in solvent (water: ACN, 50:50). Linearity range was observed in the concentration range 0.5-4.0 µg/mL with the linearity equation $y = 0.247x + 0.003$ with correlation coefficient 0.9999. The method is validated for specificity, accuracy, precision, linearity, repeatability and robustness as per ICH guideline Q2 (R1) for method validation. The limit of detection and quantitation were found to be 0.015µg/ml and 0.047µg/ml.

The optimization of mobile phase was done with different trials by using different ratio of water: ACN. The method is based on reverse phase chromatography using ZORBAX Eclipse XDB C_{18} (150×4.6 mm) 5µ particle size column. The column was maintained at 30°C throughout the analysis. The flow rate was maintained 1mL/min with injection volume 10µl.

Forced degradation studies were carried out according to ICH Guidelines. The objective of the study was to find out the likely degradation products which in turn help degradation pathway and intrinsic stability of the molecule. Stability indicating capability is established by forced degradation experiment of Agomelatine to acid, alkali, neutral, oxidation, thermal and photo degradation conditions. The peaks of degradation samples were pure and well resolved with the resolution of more than 2.0. The labelling of the peak is according to the similar UV spectra of the degradation peaks observed from the PDA detector. The linear regression analysis data for the calibration plots showed good linear relationship with concentration range of 10-40 µg/mL with the linearity equation $y = 136.1x + 88.20$ with the correlation co-efficient of 0.9999. The method is validated for specificity, accuracy, precision, linearity, repeatability and robustness as per ICH guideline Q2 (R1) for method validation. The limit of detection and quantitation were found to be 0.019µg/ml and 0.059µg/ml. The percentage of degradation in Agomelatine API and its tablet does not have much difference. Additionally, the degradation pattern is also found to be the similar as that of the Agomelatine API.

Conclusion

Hence, developed UV Spectrophotometric method was accurate, precise, rapid and cost-efficient for determination of Agomelatine in pharmaceutical dosage form. High %Recovery shows that the method is free from the interference from excipients used in the commercial pharmaceutical preparations. Hence, it can be successfully applied for routine estimation for Agomelatine in quality control laboratories. The results of validation parameters are satisfactory and the level indicates the accuracy of proposed method for estimation of Agomelatine in pharmaceutical dosage forms.

The RP-HPLC method developed for the quantification of Agomelatine is fully validated as per the ICH Guidelines, thus indicating general applicability of the method for routine analysis of formulation those marketed in regulated countries. The proposed method is simple, accurate, precise and specific and has the ability to separate the drug Agomelatine, its degradation products and excipients from each other in the tablet dosage form. Sample solution stability was established over the period of 36 hours. The degradation profile of the Agomelatine API is observed identical with its tablet formulation. The simplicity of the method allows the method for the application in the laboratory for routine quality check as well as for the stability studies for the formulated product. Overall, method provides high throughput solution for the determination of Agomelatinein the tablet with excellent selectivity, precision and accuracy.

Comparison of UV and RP-HPLC Data by paired t-test:

Sr.No.	Parameter	Pharmaceutical Formulation (Agomelatine)	
1	Mean	UV	RP-HPLC
		99.88	100.80
2	Variance	0.1408	0.0382
3	T Stat(Cal)	0.033	
4	t critical one tail	2.92	
5	t cal < t crit	YES	
6	Null Hypothesis	PASS	

Table 9.1 : **Comparison of UV and RP-HPLC Data by paired t-test**

CHAPTER 10

FUTURE PERSPECTIVES

The future experiments for the drug Agomelatine can be listed as:

- The degradation studies with liquid chromatography mass spectrophotometer (LC/MS) instrument using the developed mass-compatible method for the characterization of all the degradation peaks in the forced decomposition studies.
- Isolation of the degradation sample after the forced degradation studies using preparative HPLC method for the structural elucidation of the Agomelatine
- Development of complete degradation pathway and the impurity profiling for Agomelatine with the help of different hyphenated analytical techniques
- Different new analytical techniques like HPTLC, Raman spectroscopy, Near-IR spectroscopy and Spectrofluorometry can be developed.

CHAPTER 11

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