QUANTITATIVE ESTIMATION, ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR DRUGS ACTING ON CENTRAL NERVOUS SYSTEM

A dissertation submitted to

THE TAMILNADU Dr.M.G.R MEDICAL UNIVERSITY

CHENNAI- 600 032

In partial fulfillment of the requirements for the award of degree of

MASTER OF PHARMACY

IN

PHARMACEUTICAL ANALYSIS

SUBMITTED

BY

VEHKATESH SURISETTY

(Reg. No. 261230963)

Under the guidance of

Dr.P.Dheen Kumar, M.Pharm., Ph.D.,

DEPARTMENT OF PHARMACEUTICAL ANALYSIS

EDAYATHANGUDY.G.S PILLAY COLLEGE OF PHARMACY

NAGAPATTINAM-611002

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CERTIFICATE

This is to certify that the dissertation entitled **"QUANTITATIVE ESTIMATION, ANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR DRUGS ACTING ON CENTRAL NERVOUS SYSTEM"** submitted by VEHKATESH SURISETTY(Reg No: 261230963) in partial fulfillment for the award of degree of Master of Pharmacy to the Tamilnadu Dr. M.G.R Medical University, Chennai is an independent bonafide work of the candidate carried out under my guidance in the Department of Pharmaceutical Analysis, Edayathangudy.G.S Pillay College of Pharmacy during the academic year 2013-2014.

Place: Nagapattinam

Date:

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INTRODUCTION

A drug[1] may be defined as substance meant for diagnosis, cure, mitigation, prevention and treatment of disease in human beings or animals, for altering any structure or function of the body of human beings or animals[2]. Pharmaceutical chemistry[3-6] is a science that makes use of general laws of chemistry to study drugs i.e. their preparation, chemical nature, composition, structure, influence on an organism, the methods of quality control and the conditions of their storage etc. The family of drugs may be broadly classified as

1. Pharmacodynamic agents
2. Chemotherapeutic agents

Pharmacodynamic agents[7] refer to a group of drugs which stimulates or depress various functions of the body so as to provide some relief to the body in case of body abnormalities without curing the disease.

Chemotherapeutics agents are drugs, which are selectively more toxic to the invading organisms without causing harmful effect to the host. E.g. Antimalarial, antibacterial, antifungal agents

Every country has legislations[8] on bulk drug and their pharmaceutical formulations that sets standards and obligatory quality indices for them. These regulations are presented in separate articles relating to individual drugs and are published in the form of book called "Pharmacopoeia" [e.g. IP[9], BP[10] and Martindale Extra Pharmacopoeia[11] (MEP)]

Pharmaceutical Analysis[12-15] plays a very significant role in quality[16] control of pharmaceuticals through a rigid check on raw materials used in manufacturing of formulations and on finished products. It also plays an important role in building up the quality products through in process quality control. Pharmaceutical analysis is the application of principles of analytical chemistry to drug analysis. The analytical chemistry[17-20] may be defined as the science of developing sensitive, relative and accurate methods for determining the composition of materials in terms of elements or compounds which they contain**.** These

analytical results have played critical roles from the understanding of basic science to a variety of practical applications such as biomedical applications, environmental monitoring, quality control of industrial manufacturing and forensic science.

The methods of estimation of drugs are divided into physical, chemical, physicochemical and biological ones. Physico-chemical and physical methods are used the most. Physical methods of analysis involve the study of the physical properties of a substance. They include determination of solubility, transparency or degree of turbidity, colour density, specific gravity (for liquids), moisture content, melting, freezing and boiling points. Physicochemical methods[21-23] are used to study the physical phenomenon that occurs as result of chemical reactions. Among the physicochemical methods, the most important are optical (refractometry, polarimetry including photocolourimetry and spectrophotometry covering UV-visible and IR regions nephelometry or turbidimetry) and chromatographic (column, paper, thin-layer[24], gas liquid[25-26], HPLC[27-28]) methods. The number of new drugs is constantly growing. This requires new methods for controlling the quality.

Types of analytical chemistry[29-30]

Traditionally, analytical chemistry has been split into two main types, qualitative and quantitative:

There are main two types of chemical analysis.

1. Qualitative (identification)

2. Quantitative (estimation)

1. Qualitative analysis is performed to establish composition of natural/synthetic substances. These tests are performed to indicate whether the substance or compound is present in the sample or not. Various qualitative tests are detection of evolved gas, formation of precipitates, limit tests, colour change reactions, melting point and boiling point test etc.

2. Quantitative analytical techniques are mainly used to quantify any compound or substance in the sample. These techniques are based on

(a) The quantitative performance of suitable chemical reaction and either measuring the amount of reagent added to complete the reaction or measuring the amount of reaction product obtained

(b) The characteristics movement of a substance through a defined medium under controlled conditions

(c) Electrical measurement

(d) Measurement of some spectroscopic properties of the compound.

Different techniques of Analysis

1. Methods based on chemical analysis [31-33]

These methods are based on traditional method of analysis and may be divided as:

(i) Titrimetric
(ii) Gravimetric
(iii) Volumetry

i. **Titrimetric Analysis**[34]

In this technique the substance to be determined is allowed to react with an appropriate reagent added as a standard solution and the volume of solution needed for completion on reaction is determined. Following are the types of titrimetric analysis:

- Neutralization (acid-base) reactions.
- Complexometric titrations
- Precipitation titrations
- Oxidation- reduction titrations
- Non aqueous titrations

ii. **Gravimetric Analysis**[35]**:**

In this technique substance under determination is converted into an insoluble precipitate which is collected and weighed. In a special case of gravimetric analysis,

electrolysis of the substance is carried out and the material deposited on one of the electrodes is weighed. This technique is called as electrogravimetry.

iii. **Volumetry Analysis:**

It is concerned with measuring the volume of gas evolved or absorbed in a chemical reaction.

2. Electrical Methods of Analysis[36-37]**:** These involve the measurement of current voltage or resistance in relation to the concentration of a certain species in a solution. These methods are of following types:

a) **Voltametry:**

It is the measurement of current at a microelectrode at a specified voltage.

b) **Coulometry:**

It is the measurement of current and time needed to complete an electrochemical reaction or to generate sufficient material to react completely with a specified reagent.

c) **Conductometry:**

It is the measurement of electrical conductivity of a solution. The ionic reactions in which there is a sudden change in conductance after completion of reaction, can act as a basis of conductometric titration method.

d) **Potentiometry:**

It is the measurement of the potential of an electrode in equilibrium with an ion to be determined.

3. Optical methods of analysis[38-40]

The optical methods are basically of two types:

i. Absorption methods

ii. Emission methods.

i. Absorption methods:

Absorption spectroscopy refers to spectroscopic techniques that measure the absorption of radiation, as a function of frequency or wavelength, due to its interaction with a sample. The sample absorbs energy, i.e., photons, from the radiating field. The intensity of the absorption varies as a function of frequency and this variation is the absorption spectrum. Absorption spectroscopy is performed across the electromagnetic spectrum. Absorption spectroscopy is employed as an analytical chemistry tool to determine the presence of a particular substance in a sample and in many cases, to quantify the amount of the substance present.

Absorption methods are usually classified according to wavelength involved:

i. Visible spectrophotometry

ii. Ultraviolet spectrophotometry

iii. Infrared spectrophotometry

Visible spectrophotometry:

The method of analysis is based on measuring the absorption of monochromatic light by colored compounds in the visible path of the spectrum (370-800 nm). If the analytes are colorless, they are converted into colored compounds by reaction with a suitable chromogenic reagent. In this case, the majority of colored compounds are complexes or complex ligands. The later must be stable and have a constant composition and high color intensity. The photometric methods of analysis are based on the Bouger-Lambert-Beer's law, which establishes that absorbance by a solution is directly proportional to the concentration of the analyte. The fundamental principle of operation of spectrophotometer covering visible region consists in that light of definite interval of wavelength passes through the radiant energy into electrical energy measured by galvanometer.

The absorption of light by analytes is due to the presence of chromophores in their molecules, which are specific portions of molecules can absorb radiant energy in the UV or visible region. They include unsaturated functional groups. Every functional group in a molecule of a substance is characterized by the absorption of light in a definite region of the spectrum and this property is used for the identification and quantification of a substance in a drug. In addition to chromophores, a molecule may contain one or more saturated functional groups that themselves do not absorb in the UV/visible region being scanned, but can affect the behaviour of the chromophores that are conjugated with these groups are called auxochrome (e.g. SH, NO_2, OH), which usually cause absorption by a chromophores at higher wavelength and at a longer value of the absorptivity than found for the given chromophores itself.

Absorption spectrum

The absorption spectrum is a graphical representation of the amount of light absorbed by a substance at definite wavelengths. To plot absorption curve, the value of the wavelength (λ) are laid off along the axis of abscissas and the values of the absorbance along the axis of the ordinates. A characteristic of an absorption spectrum is a position of the peaks (maxima) of light absorption, which is determined by the absorptivity at definite wavelengths.

Beer's law plot

A standard specimen of the analyte is taken and the solutions of it with known concentrations are prepared. The absorbance of all the solutions are measured at a definite wavelength (λ_{max}) and the calibration curve is plotted by laying off the known concentrations along the axis abscissas and the absorbances corresponding to them along the axis ordinates. The calibration curve is used to determine the unknown concentration of the analyte in its solutions.

A feature of organic drugs is the presence of functional groups in their molecules i.e., reactive atoms or groups of atoms determined by chemical reactions. Functional groups determine the way of analysing organic drugs because they are responsible for the properties of substance and determine the identification reactions and the methods of quantitative determination of drugs. Knowing the reactions for detecting functional groups, one can easily and conscientiously analyze any organic drug with a complicated structure. There are several drug molecules, which are poly functional in nature, i.e., simultaneously contain two or more functional groups[41-44].

ii. Emission Method

In **emission** method sample is subjected to heat or electrical treatment so that the atoms are raised to excited states causing them to emit-energy; and the intensity of this emitted energy is measured. The emission spectroscopy includes flame photometry and fluorimetry. In emission spectroscopy the sample is subjected to flame, electric arc or spark plasma and the light emitted is examined. Flame photometry involves the solution of the sample, injected into a flame while in fluorimetry a suitable substance in solution is excited by irradiating with visible or ultraviolet radiation.

Chromatography[45-48]

Introduction:

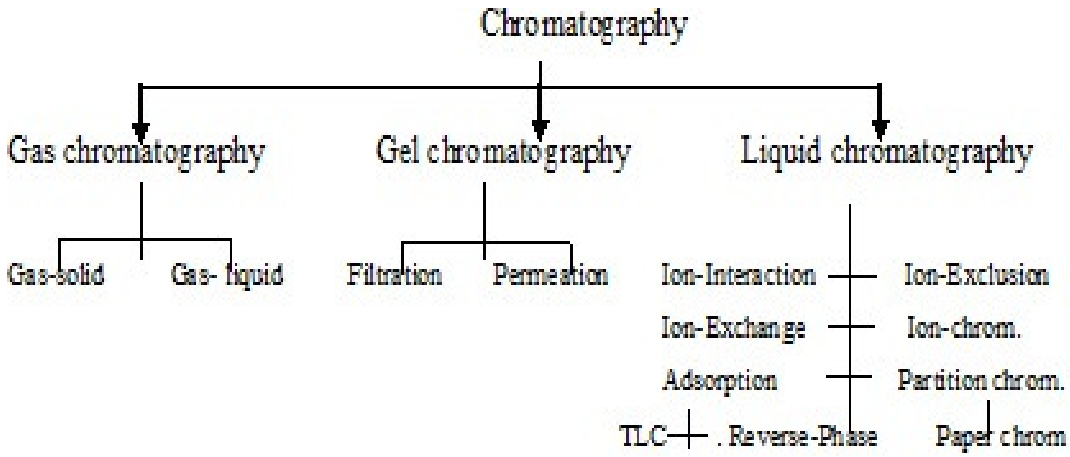
Chromatography (from Greek: *chroma*, colour and: *grafein* to write) is the collective term for a family of laboratory techniques for the separation of mixtures. It involves passing a mixture dissolved in a "mobile phase" through a stationary phase, which separates the analyte to be measured from other molecules in the mixture and allows it to be isolated.

Chromatography may be preparative or analytical. Preparative chromatography seeks to separate the components of a mixture for further use (and is thus a form of purification).

Analytical chromatography normally operates with smaller amounts of material and seeks to measure the relative proportions of analytes in a mixture. The two are not mutually exclusive.

Types of Chromatography[49-51]

Chromatography can be divided into three subsections namely gas, gel and liquid chromatography. Gas chromatography is used for the analysis of volatile samples, gel chromatography for non-volatile samples with a molecular weight smaller than 2000.



Gas Chromatography (GC) [52-54]

Gas chromatography is a chromatographic technique that can be used to separate organic compounds that are volatile. A gas chromatography consists of a flowing mobile phase, an injection port, a separation column containing the stationary phase, a detector, and a data recording system. The organic compounds are separated due to differences in their partitioning behaviour between the mobile gas phase and the stationary phase in the column.

High-performance liquid chromatography (HPLC) [55-56]

In the field of analytical chemistry high performance liquid chromatography (HPLC) is considered by many to be most exciting and dynamic technique of past decade. The typical HPLC separation is based on the selective distribution of analytes between a liquid mobile phase and an immiscible stationary phase. The sample is first introduced by means of an

injection port into the mobile phase stream that is delivered by a high-pressure pump. Next, the components of this sample mixture are separated on the column, a process monitored with a flow-through detector as the isolated components emerge from the column.

General Methodology for Analytical Method Development

Development of a method[57-58]:

In developing a quantitative method for determining an unknown concentration of a given substance by absorption spectrophotometry, the first step will be the selection of analytical wavelength which can be chosen either from literature or experimentally by means of a scanning spectrum in the UV-Visible region. In order to enhance the sensitivity of the method and signal to noise ratio, the wavelength of maximum absorbance is chosen as analytical wavelength.

After selection of the wavelength, the colour developing reagent and the absorbing product must be stable for a considerable period of time. Always the preparation of standards and unknown should be on a definite time schedule.

Optimization of analytical method[59]:

The bases of the spectrophotometric methods, in the present investigation are

(a) Oxidative coupling

(b) Oxidation followed by complex formation

(c) Diazotization and coupling

(d) Complex formation.

In each type of reaction, the yield of the coloured species whose absorbance is measured and thus the sensitivity of the method, rate of color formation and stability are affected by the concentration of the reagent in the solution. The nature of the solvent, the temperature, the pH of the medium, order of addition of reactants and waiting periods also affect the above parameters. For simple systems having no interaction between variables, the

one variable at time (OVAT) strategy appears to be simple, efficient and effective to establish the optimum conditions. The OVAT approach requires all variable but one to be held constant while a univariate search is carried out on the variable of interest.

Calibration:

Calibration is one of the most important steps in drug analysis. A good precision and accuracy can only be obtained when good calibration procedure is used. In spectrophotometric methods the concentration of a sample cannot be measured directly, but is determined using another physical measuring quantity, "Y" (absorbance of a solution). An unambiguous empirical or theoretical relationship can be shown between this quantity and concentration of the analyte.

For the majority of analytical techniques the analyst uses the calibration equation.

$$Y= a + bX$$

In calibration, univariate regression is applied, which means that all observations are dependent upon a single variable "X".

The method of least squares[60]:

Least squares regression analysis is used to describe the relationship between signal and concentration. All models describe the relationship between response (Y) and concentration (X) can be represented by general function.

$$Y= f(X, a_1, b_1.....b_m)$$

Where a_1, b_1.....b_m are the parameters of the function

We adopt the convention that 'X' values relate to the controlled or independent variable and the 'Y' values to the dependent variable. This means that 'X' value has no error. On the condition that errors made in preparing the standards are significantly smaller than the measuring errors this assumption is realistic in calibration problems. The values of

unknown parameter a_1, b_1.....b_m must be estimated in such a way that the model fits the experimental data points as far as possible.

The true relationship between X and Y is considered to be given by a straight line. The relationship between each observation pair (X_i, Y_i) can be represented as

$$Y_i=\alpha + \beta X_i + e_i$$

The signal Y_i is composed of a deterministic component predicted by linear model and a random component e_i. One must now find the estimates 'a' and 'b' of the true values and α and β which are constants. This is done by calculating values 'a' and 'b' for which $\sum e_i^2$ is minimal. The component e_i represents the difference between the observed Y_i values by the model. The e_i are called the residuals, 'a' and 'b' are the intercept and slope respectively. The equation given for slope and intercept of the line are as follows.

$$\text{Slope (b)} = \frac{n\sum_i X_i Y_i - \sum_i X_i \cdot \sum_i Y_i}{n\sum_i X_i^2 - [\sum_i X_i]^2}$$

$$\text{Intercept (a)} = \frac{\sum_i Y_i \sum_i X_i^2 - \sum_i X_i \cdot \sum_i X_i Y_i}{n\sum_i X_i^2 - [\sum_i X_i]^2}$$

Correlation coefficient (r)

The correlation coefficient r (x, y) is more useful to express the relationship of the chosen scales. To obtain a correlation, the covariance is divided by the product of the standard deviation of x and y.

$$r = \frac{\left[\sum_{i=1}^{n}\left(X_i-\overline{X}\right)\left(Y_i-\overline{Y}\right)\right]}{(n-1)} \Bigg/ \frac{\left[\sum_{i=1}^{n}\left(X_i-\overline{X}\right)^2\sum_{i=1}^{n}\left(Y_i-\overline{Y}\right)^2\right]}{(n-1)^2}$$

Where $\overline{X}, \overline{Y}$ are the arithmetic means of X and Y respectively.

Selectivity of the method

The determination of an analyte may be disturbed by matrix and interference effect. Some of the excipients, incipient and additives present in pharmaceutical formulations may sometimes interfere in the assay of the drug and in such instances appropriate separation procedure is to be adopted initially. The selectivity of the method ascertained by studying the effect of a wide range of excipients and other additives usually present in the pharmaceutical formulation on the determinations under optimum conditions.

In the initial interference studies, a fixed concentration of the drug is determined several times by the optimum procedure in the presence of suitable (1 to 100 fold) molar excess of foreign compounds under investigation and its effect on absorbance of solution is noticed. The foreign compound is considered to be non-interfering if at these concentrations, it constantly produces an error less than 3% in the absorbance produced in the pure sample solution.

Linearity and sensitivity of the method

Knowledge of the sensitivity of the color is important and the following terms are commonly employed for expressing the sensitivity. According to the Beer's law

$$A = \log \frac{\text{Intensity of incident radiation}}{\text{Intensity of transmitted light}} = \varepsilon.C.T$$

The absorbance (A) is proportional to the concentration (C) of absorbing species if absorptivity (ε) and thickness of the medium (t) are constant. When 'C' is in moles per liter,

the constant ε is called the molar absorptivity. Beer's law and ε_{max} values are expressed as μg/ml and $mole^{-1}cm^{-1}$ respectively.

Sandell's sensitivity[61] refers to the number of μg of the drug determined, converted to the colored product, which in a column solution of cross section $1cm^2$ shows an absorbance 0.001 (expressed as $\mu g\ cm^{-2}$)

Ringbom's plot[62]

The relative concentration error depends inversely upon the product absorbance and transmittance. The relative error increases at the extremes of the transmittance (T) scale. The slope of plot 'C' versus T, i.e. Ringbom's plot gives relative coefficient (i.e. plot of log C α T). The main limitation of the ringbom's plot is that it provides no information concerning the concentration range of good precision unless it is combined with ΔT versus T relation. The above expression is valid whether or not Beer's law is valid.

Recovery experiments (standard addition method)

A known amount of the constituent being determined is added to the sample which is analyzed for the total amount of constituent present. The difference between the analytical results for samples with and without the added constituent gives the recovery of the amount of added constituent. If recovery is satisfactory our confidence in the accuracy of the procedure will be enhanced.

Usually, recovery studies are performed while proceeding for pharmaceutical formulations; known amounts of an analyte are spiked at different levels into a sample matrix, which was already analyzed. The concentration of the analyte in the original sample may then be determined mathematically.

$$\% \text{ Recovery} = \left[\frac{N\left(\sum XY\right)-\sum X.\sum Y}{N\left(\sum X^2\right)-\left(\sum X\right)^2}\right]\times 100$$

X= amount of standard drug

Y= amount of drug found by the proposed method

N= Number of observations

ANALYTICAL METHOD VALIDATION AS PER ICH GUIDELINES[63-66]

Method validation is the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Methods need to be validated or revalidated.

- Before their introduction into routine use.
- Whenever the conditions change for which the method has been validated, e.g., instrument with different characteristics.
- Whenever the method is changed, and the change is outside the original scope of the method.

Method validation is completed to ensure that an analytical methodology is accurate, specific, reproducible and rugged over the specified range that an analyte will be analyzed. Method validation provides an assurance of reliability during normal use, and is sometime referred to as "the process of providing documented evidence that the method does what it is intended to do." Regulated laboratories must perform method validation in order to be in compliance with FDA regulations.

For method validation, these specifications are listed in USP Chapter <1225>, and can be referred to as the "Eight Steps of Method Validation". These terms are referred to as "**analytical performance parameters**", or sometimes as "analytical figures of merit."

In response to this situation, one of the first harmonization projects taken up by the ICH was the development of a guideline on the "Validation of Analytical Methods: Definitions and Terminology[67]."

METHOD VALIDATION

The developed methods were validated by following steps

- Accuracy

- Precision
- Specificity
- Limit of quantitation
- Limit of detection
- Linearity and range
- Ruggedness and
- Robustness

Accuracy:

It is defined as closeness of agreement between the actual (true) value and mean analytical value obtained by applying a test method number of times. Accuracy of an analytical method is determined by systematic error involved. The accuracy is acceptable if the difference between the true value and mean measured value does not exceed the RSD values obtained for repeatability of the method.

The parameter provides information about the recovery of the drug from sample and effect of matrix, as recoveries are likely to be excessive as well as deficient.

To document accuracy, the ICH guideline on methodology recommends collecting data from a minimum of nine determinations over a minimum of three concentration levels, covering the specified range (for example, three concentrations, three replicates each).

Accuracy is calculating the percentage recovery by the assay of the known amount of analyte in the sample or as the difference between the mean and the accepted true value, together with confidence intervals.

For assay method, spiked samples are prepared in triplicate at three intervals over a range of 50-100% of the target concentration. Potential impurities should be added to the matrix to mimic impure samples. The analyte levels in the spiked samples should be determined using the same quantitation procedure as will be used in the final method

procedure (i.e. same levels of standards and same number of samples and standard injections).

Precision:

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple sampling of homogenous sample. Precision is the measure of the degree of repeatability of an analytical method under normal operation and is normally expressed as the percent relative standard deviation for a statistically significant number of samples. According to the ICH, precision should be performed at three different levels: *repeatability, intermediate precision, and reproducibility.*

The precision of an analytical method is determined by assaying a sufficient number of aliquots of a homogenous sample to be able to calculate statistically valid estimates of standard deviation or relative standard deviation. The ICH documents recommended that the repeatability should be assessed using a minimum of nine determinations covering the procedure (i.e. three concentration and three replicates of each concentrations using a minimum of six determinations at 100% of the test concentrations)

In the case of *instrument precision*, six replicates of the standard solution are made for the test performance of the chromatographic instrument.

In the case of *method precision*, six replicates from the same batch are analyzed for the assay and dissolution parameters and observing the amount of scatter in the results. An example of precision criteria of an assay method is that the instrument precision RSD should not be more than 2.0%.

Repeatability is the results of the method operating over a short time interval under the same conditions (inter-assay precision). It should be determined from a minimum of nine determinations covering the specified range of the procedure (for example, three levels, three

repetitions each) or from a minimum of six determinations at 100% of the test or target concentration.

Intermediate precision is the results from within lab variations due to random events such as different days, analysts, equipment, etc. In determining intermediate precision, experimental design should be employed so that the effects (if any) of the individual variables can be monitored.

Reproducibility refers to the results of collaborative studies between laboratories. Documentation in support of precision studies should include the standard deviation, relative standard deviation, coefficient of variation, and the confidence interval.

Specificity:

It is the ability of an analytical method to assess unequivocally the analyte of interest in the presence of components that may be expected to be present, such as impurities, degradation products and matrix components. In case of the assay, demonstration of specificity requires that the procedure is unaffected by the presence of impurities or excipients. In practice, this can be done by spiking the drug substances or product with appropriate levels of impurities or excipients and demonstrating that the assay is unaffected by the presence of these extraneous materials. If the degradation product impurity standards are unavailable, specificity may be demonstrated by comparing the test results of samples containing impurities or degradation products to a second well-characterized procedure. These comparisons should include samples stored under relevant stress conditions (e.g. light, heat humidity, acid/base hydrolysis, oxidation, etc.).

Limit of Detection:

The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected, not quantitated. It is a limit test that specifies whether or not an analyte is above or below a certain value. It is expressed as a concentration at a specified signal-to-noise ratio, usually two or three-to-one. The ICH has recognized the signal-to-noise

ratio convention, but also lists two other options to determine LOD: visual non-instrumental methods and a means of calculating the LOD. Visual non-instrumental methods may include LOD's determined by techniques such as thin layer chromatography (TLC) or titrations. LOD's may also be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) at levels approximating the LOD according to the formula: LOD = 3.3(SD/S). The standard deviation of the response can be determined based on the standard deviation of the blank, on the residual standard deviation of the regression line, or the standard deviation of y-intercepts of regression lines. The method used to determine LOD should be documented and supported and an appropriate number of samples should be analyzed at the limit to validate the level.

Limit of Quantitation:

The Limit of Quantitation (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. Like LOD, LOQ is expressed as a concentration, with the precision and accuracy of the measurement also reported. Sometimes a signal-to-noise ratio of ten-to-one is used to determine LOQ. This signal-to-noise ratio is a good rule of thumb, but it should be remembered that the determination of LOQ is a compromise between the concentration and the required precision and accuracy. That is, as the LOQ concentration level decreases, the precision increases. If better precision is required, a higher concentration must be reported for LOQ. This compromise is dictated by the analytical method and its intended use. The ICH has recognized the ten-to-one signal-to-noise ratio as typical, and also, like LOD, lists the same two additional options that can be used to determine LOQ, visual non-instrumental methods and a means of calculating the LOQ. The calculation method is again based on the standard deviation of the response (SD) and the slope of the calibration curve (S) according to the formula: LOQ = 10(SD/S).

Linearity and Range:

Linearity is the ability of the method to elicit test results that are directly proportional to analyte concentration within a given range. Linearity is generally reported as the variance of the slope of the regression line. Range is the interval between the upper and lower levels of analyte (inclusive) that have been demonstrated to be determined with precision, accuracy and linearity using the method as written. The range is normally expressed in the same units as the test results obtained by the method ICH25 recommended that, for the establishment of linearity, a minimum of five concentrations. It is also recommended that the following minimum specified range should be considered. For assay of a drug substance or a finished product 80-120% of the test concentration should be taken. For an impurity test, the minimum range is from the reporting level of each impurity, to 120% of the specification. (For toxic or more potent impurities, the range should be commensurate with the controlled level.)

Acceptability of the linearity data is often judged by examining the correlation co-efficient and y-intercept of the linear regression line for the response versus concentration plot. The correlation coefficient of >0.999 is generally considered as evidence of acceptable fit of the data to the regression line. The y-intercept should be less than a few percent of the response obtained for the analyte at to target level.

Ruggedness:

Ruggedness, according to the USP, is the degree of reproducibility of the results obtained under a variety of conditions, expressed as %RSD. The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions such as different laboratories, different analysts, different instruments, different lots of reagents, different elapsed assay times, different assay temperatures, different days, etc.

Robustness:

Robustness is the capacity of a method to remain unaffected by small deliberate variations in method parameters. The robustness of a method is evaluated by varying method parameters such as percent organic, pH, ionic strength, temperature, etc., and determining the effect (if any) on the results of the method. As documented in the ICH guidelines, robustness should be considered early in the development of a method. In addition, if the results of a method or other measurements are susceptible to variations in method parameters, these parameters should be adequately controlled and a precautionary statement included in the method documentation.

The robustness of the methods was determined by performing the assay of the triplicate by deliberately alternating parameters and that the results are not influenced by different changes in the above parameters

Change in column temperature $\pm 5^0$C

Change in flow rate ±10%.

Change in organic phase ± 2%.

Change in pH ± 0.2.

The system suitability and the precision of the assay were evaluated for the respective condition. The robustness of an analytical procedure is the measure of its capability to remain unaffected by small, but deliberate, variation in method parameters and providers an indication of its reliability during normal usage.

Chromogenic reagents used in the present investigation.

Functional groups present in organic drugs determine the way of analyzing them because they are responsible for the properties of substances and determine the identification reaction and the methods of quantitative determination of drugs. Knowing the reactions for

detecting functional groups, one can easily analyze any organic drug with a complicated structure. In the present investigation, few visible spectrophotometric methods have been developed for Pregabalin and Zolpidem by developing colour in each case with, appropriate reagent. The analytically useful functional groups in the drug have not been exploited completely in developing the new visible spectrophotometric method and so, the drugs have been selected in the present investigation.

Different type of reagents like Folin's, Sanger's, Brady's, Para chloranilic acid, Gibb's, MBTH, BM reagent were used in the present investigation for developing visible spectrophotometric methods.

1, 2- Napthoquinone-4-sulphonate (NQS reagent) [68-69]

NQS reagent is also called as Folin's reagent. This reagent reacts with the compounds containing amine group**.** In the present study due to the presence of amine as chromophoric group in the Pregabalin molecule, derivatization of the compound was attempted with this reagent, as a result colored complex has been formed which was estimated spectrophotometrically. This reagent was generally prepared in water.

1-Flouro-2, 4-dinitrobenzene[70-71]

1-Flouro-2, 4-dinitrobenzene is also called as Sanger's reagent. Sanger's reagent reacts with the compounds containing amine group. The reaction of pregabalin with Sanger's reagent resulted in the formation of Zwitter-ion-like structure due to the presence of the carboxylic acid group adjacent to amino group. Reagent was prepared in methanol for the present study. Reagent was highly skin irritant and stable up to seven days if stored in the refrigerator. Care must be taken while using it.

2, 4-Dinitrophenyl hydrazine[72-73]

2, 4-Dinitrophenyl hydrazine reagent is also called as Brady's reagent. Dinitrophenylhydrazine is relatively sensitive to shock and friction; it is a shock explosive so

care must be taken with its use. It is red to orange solid, usually supplied wet to reduce its explosive hazard. It is a substituted hydrazine, and is often used for the determination carbonyl groups associated with aldehydes and ketones. The reagent was generally prepared in water.

Parachloranilic acid[74-75]

p-Chloranilic acid mainly involves in the charge transfer reactions. P-CA acts as a π acceptor. The interaction of drug with π-acceptor (p-CA) at room temperature was found to yield colored charge transfer complex. In polar solvents, complete electron transfer from drug as an electron donor, to the acceptor moiety takes place resulting in the formation of intensely colored radical anions. The reagent was prepared in methanol for the present investigation.

2, 6 Dichloroquinone chlorimide[76-77]

2, 6 Dichloroquinone chlorimide is also called as Gibb's reagent. Gibb's reagent mainly reacts with phenols, primary amines, secondary amines, aliphatic amines. For the present study the reagent was prepared in methanol.

3-Methyl 2-benzathiozolinone hydrazone[78-80]

MBTH is synthesized by Besthron. MBTH can react with carbonyl compounds and compounds containing amine group. It also forms a strongly electrophillic diazonium salt when acted upon by an oxidizing agent. Ferric chloride has been mostly used as the oxidizing agent for the determination of amines. For the present study the reagent was prepared in distilled water.

Bratton Marshal reagent (BMR) [81-82]

Bratton Marshal reagent is also called as N-(1-naphthyl) ethylene diamine dihydrochloride. It was white to light tan or grey crystalline solid or off white powder. It was prepared by dissolving in water. It was light sensitive and hygroscopic. It mainly reacts with the compounds containing amine as the functional group.

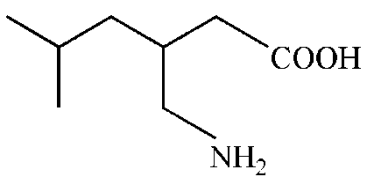
Central nervous system (CNS) acting drugs are widely used to treat various diseases like Parkinsonism, Schizophrenia, Mania, Epilepsy, Insomnia, Depression, Anxiety, Meningitis etc. A number of novel drugs like Pregabalin, Zolpidem, Galantamine, Levetiracetam, Premipexole were reaching the market as an attempt to treat CNS diseases and disorders. In the present study we have been selected Pregabalin and Zolpidem for the quantitative determination. The main purpose of selecting those drugs was according to the literature survey very few chromatographic and spectrophotometric method were developed and still there was lot of scope to develop many analytical methods for the determination of those drugs.

DRUG PROFILE

PREGABALIN

IUPAC Name : [S-[+]-3-isobutyl GABA or (S)-3-(amino methyl)-5-methylhexanoic acid.

Structural Formula :



Chemical formula : $C_8H_{17}NO_2$

Molecular Mass **:** 159.23 g/mol.

Description **:** Pregabalin is a white to off-white crystalline solid

Solubility **:** Freely soluble in water and methanol

Melting point **:** 127-134^{0}C

Category **:** Pregabalin is an anticonvulsant and used in treatment of diabetic neuropathy.

Storage **:** Pregabalin should be kept in well closed container, protected from light.

Indication **:** For management of neuropathic pain associated with diabetic peripheral neuropathy and postherpeutic neuralgia.

Mechanism of Action: Pregabalin binds with high affinity to the α-2-delta site (an auxiliary subunit of voltage-gated calcium channels) in central nervous system tissues. Although the mechanism of action of pregabalin is unknown, results with genetically modified mice and with compounds structurally related to pregabalin (such as gabapentin) suggest that binding to the alpha2-delta subunit may be involved in pregabalin's antinociceptive and antiseizure effects in animal models. In vitro, pregabalin reduces the calcium-dependent release of several neurotransmitters, possibly by modulation of calcium channel function.

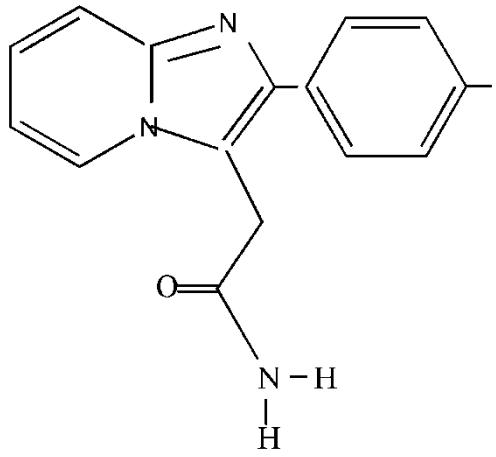
Drug Interactions **:** No pharmacokinetic interactions have been demonstrated in vivo. But some potential pharmacological interactions with opioids (pregabalin is synergistic with opioids in lower doses), benzodiazepines, barbiturates, ethanol (alcohol), and other drugs that depress the central nervous system were noticed.

Dosing : The recommended starting pregabalin dose for treating nerve pain due to diabetic neuropathy is 50 mg three times daily. The recommended starting pregabalin dose for controlling partial seizures in people with epilepsy is 75 mg twice daily or 50 mg three times daily.

ZOLPIDEM

IUPAC Name : N, N, 6-Trimethyl-2-p-tolyl-imidazole (1,2-a)pyridine-3-acetamide L-(+)-tartrate

Structural Formula :



Chemical formula : $(C_{19}H_{21}N_3O)_2 \cdot C_4H_6O_6$

Molecular Mass : 764.88 g/mol.

Description : Zolpidem is a white to off-white, hygroscopic, crystalline powder.

Solubility : Freely soluble in water and sparingly soluble in methanol.

Melting point : 195^0C

Category : Zolpidem is used as hypnotic.

Storage : Zolpidem should be kept in air tight container, protected from light.

Mechanism of Action **:** Due to its selective binding, Zolpidem has very weak anxiolytic, myorelaxant and anticonvulsant properties but very strong hypnotic properties. Zolpidem binds with high affinity and acts as a full agonist at the α_1 containing $GABA_A$ receptors, about 10-fold lower affinity for those containing the α_2 and α_3 - $GABA_A$ receptor subunits, and with no appreciable affinity for α_5 subunit containing receptors. ω_1 type $GABA_A$ receptors are the α_1 containing $GABA_A$ receptors and ω_2 $GABA_A$ receptors are the α_2, α_3, α_4, α_5 and α_6 containing $GABA_A$ receptors. ω_1 $GABA_A$ receptors are primarily found in the brain whereas ω_2 receptors are primarily found in the spine. Thus zolpidem has a preferential binding for the $GABA_A$-benzodiazepine receptor complex in the brain but a low affinity for the $GABA_A$-benzodiazepine receptor complex in the spine. Like the vast majority of benzodiazepine-like molecules, zolpidem has no affinity for α_4 and α_6 subunit-containing receptors. Zolpidem positively modulates $GABA_A$ receptors, probably by increasing the $GABA_A$ receptor complexes apparent affinity for GABA, without affecting desensitization or peak current. Zolpidem increases slow wave sleep and caused no effect on stage 2 sleep in laboratory tests.

Drug Interactions **:** Notable drug-drug interactions with the pharmacokinetics of zolpidem include the following drugs chlorpromazine, fluconazole, imipramine, itraconazole, ketoconazole, rifampicin, ritonavir. Interactions with carbamazepine and phenytoin can be expected based on their metabolic pathways but have not yet been studied.

There does not appear to be any interaction between zolpidem and cimetidine and rantidine.

Dosing **:** The usual dose of zolpidem in adults is 5–10 mg. For healthy adults, 10 mg is commonly recommended.

REVIEW OF LITERATURE

PREGABALIN

Rajinder Singh Gujral[83] ***et al*** reported a method for the determination of pregabalin in bulk, pharmaceutical formulations and human urine samples using a isocratic reversed phase HPLC method. Separation was accomplished on a C_{18} 5µm ODS hypersil column using a methanol, acetonitrile and 0.02M di-potassium hydrogen ortho phosphate (3: 1: 16, V/V/V) as mobile phase. The compound was eluted at a flow rate of 1 ml/min. For detection of the pregabalin 210 nm wavelength was set. Linearity range of the method was 0.75-6.00 µg/ml.

Jadhav S[84] ***et al*** reported a enantio selective high performance liquid chromatographic method, with precolumn derivitization with marfey's chiral reagent, sodium 2,4-dinitro-5-fluorophenyl-L-alamine amide for resolution of the enantiomers of a new anti epileptic drug, pregabalin, in the bulk drug. The separation was accomplished using a reversed phase ODS column with a mixture of aqueous 0.2% triethyl amine and acetonitrile (60:40) as mobile

phase. The linearity range of the method was 750 to 7,500 ng/l. Recovery of R enantiomer from bulk drug samples of pregabalin ranged from 97.5 to 101.76%.

Armagan Ona[85] ***et al*** developed two spectrofluorimetric and spectrophotometric methods for determination of pregabalin in bulk drug and capsule. Pregabalin react with 7-chloro-4-nitrobenzofurazon which is highly sensitive fluorogenic and chromogenic reagent used in many investigations. The relation between the absorbance at 460 nm and the concentration is rectilinear over the range 0.5–7.0 μg mL^{-1}. Measurement was also made spectrofluorimetrically at 558 nm after excitation at 460 nm. The fluorescence intensity was directly proportional to the concentration over the range 40–400 ng/ml. The mean recovery for the commercial capsules was 99.93% and 99.96% for spectrophotometric and spectrofluorimetric study, respectively.

Onal Armagan[86] ***et al*** reported three methods for the spectrophotometric determination of pregabalin in pharmaceutical preparations. Two methods are based on the reaction of pregabalin as n-electron donors with 2, 3-dichloro -5,6-dicyano-1,4-benzoquinone(DDQ) and 7,7,8,8 tetra cyanoquinodimethane (TCNQ) as π-acceptor to give highly colored complex species which were quantitated spectrophotometrically. The third method is based on the interaction of ninhydrin with primary amine present in the pregabalin. The reaction product was measured spectrophotometrically at 495 nm, 841 nm, and 573 nm respectively. The linearity ranges of the three methods were 2.0-3.0 g/ml, 1.5-10 g/ml, and 40.0-180.0 μg/ml. respectively.

Vermeij TAC[87] ***et al*** presented a HPLC method for the simultaneous determination of the γ-amino-n-butyric acid (GABA) derivatives, pregabalin, gabapentin and vigabatrin in human serum by HPLC. Serum is deproteinized with trichloroacetic acid and aliquots of the supernatant are precolumn derivatized with o-phtaldialdehyde (OPA) and 3-mercaptopropionic acid. The drugs are monitored using fluorescence detection. Separation is

achieved on an Alltima 3C18 column using isocratic elution. The method is linear up to at least 63 mg/l for PGB, 40 mg/l for GBP and 62 mg/l for VGB.

Berry[88] ***et al*** developed a HPLC method for assay of the new antiepileptic drug pregabalin in serum/plasma. Acetone precipitation of the drug was carried out and derivitized with picryl sulfonic acid (PSA) before chromatography on C_8 column. The linearity range of the method was 2.8-8.2 mg/L at steady state.

Vermeij TAC[89] ***et al*** developed a method for the simultaneous determination of seven antiepileptic drugs, including primidone, Phenobarbital, phenytoin, carbamazepine, lamotrigine, hydroxycarbazepine and zonisamide in serum by HPLC-diode array detector (DAD). Separation is achieved on an Alltima 3C18 analytical column using isocratic elution with a mixture of acetonitrile, methanol and phosphate buffer at 45 °C as mobile phase.

Rajinder Singh Gujral[90] ***et al*** developed a spectrophotometric method for determination of pregabalin in bulk, formulations and in human urine samples. The method is based on the reaction of drug with the mixture of potassium iodate and potassium iodide and the absorbance was measured at 353 nm.

Hesam Salem[91] studied the charge transfer reaction of pregabalin as n-electron donor with various π-acceptors: 7,7,8,8-tetracyanoquinodimethane (TCNQ), 2,3-dichlore-5,6-dicyano-1,4-benzoquinone (DDQ), 2,5-dichloro-3,6-dihydroxy-1,4benzoquinone and 2,3,5,6-tetrchloro-1,4-benzoquinone. Different colored charge-transfer complexes were obtained which are utilized in the development of spectrophotometric methods for the analysis of pregabalin in pure form as well as formulations. The linearity range of the method was 8-400 µg/ml. The recovery percentages of the method ranged from 100.19±0.83 to100.50±0.53.

Uttam Mandal[92] ***et al*** developed a bioanalytical method for determination of pregabalin in human plasma. This analytical method consists in the precipitation of plasma sample with trichloro acetic acid (20% v/v solution in water), followed by the determination of pregabalin

by an LC-MS-MS method using gabapentin as internal standard. Separation was achieved on a Gemini C18 50 mm · 2.0 mm (3 lm) column with an isocratic mobile phase consisting of methanol–water (98:2, v/v) with 0.5% v/v formic acid. Protonated ions formed by a turbo ionspray in positive mode were used to detect analyte and internal standard. The MS-MS detection was by monitoring the fragmentation of 160 ▶ 55.1 (m/z) for pregabalin and 172.2 ▶ 67.1 (m/z) for gabapentin on a triple quadrupole mass spectrometer.

Vaidya V[93] ***et al*** developed a method to quantify pregabalin in human plasma using metaxalone as the internal standard. Sample preparation involved simple protein precipitation by using acetonitrile as solvent. Chromatography was performed isocratically on thermo hypurity C18 5 μm analytical column. The assay of pregabalin was linear calibration curve over the range 10.000–10000.000 ng/ml.

Rasha Abdel[94] ***et al*** developed three spectrofluorimetric and spectrophotometric methods for the determination of pregabalin. Pregabalin as a primary amine reacts with fluorescamine to yield a fluorescent product, with 2,4-dinitrofluorobenzene and 2,3,5,6-tetrachloro-1,4-benzoquinone in aqueous alkaline buffered media to form colored products which could be measured spectrophotometrically. The optimum conditions for each reaction were ascertained and the methods were applied for the determination of pregabalin over the concentration range of 20-280 ng/ml and 1 – 7 μg/ml for spectrofluorimetry and spectrophotometry respectively with good correlation (≥0.999).

Kannapan N[95] ***et al*** developed a RP-HPLC method for determination of pregabalin and methylcobalamine combined in capsule dosage form. Separation was achieved isocratically using Waters allaiance 2695 seperation module, C18 column (250 x 4.6 mm, 5mcg/ml) at temperature 40°C. Flow rate selected was 1ml/min using UV visible PDA detector at 210 nm. Mobile phase was prepared using ammonium dihydrogen-o-phosphate (buffer 6.0), acetonitrile and methanol in the ratio of 75:15:10 which gave better resolution and sensitivity.

The method was found to be linear in the range of 3200-4800 mcg/ml and 16-24 mcg/ml for pregabalin and methylcobalamine.

Rajinder Singh Gujral[96] ***et al*** developed a UV spectrophotometric method for the determination of pregabalin in bulk, pharmaceutical formulations and in human urine samples. The method was linear in the range of 0.5–5.0 μg/ml. The absorbance was measured at 210 nm.

ZOLPIDEM

Hempel G[97] ***et al*** **reported a method** for the determination of zolpidem and its main metabolites in urine without extraction using capillary electrophoresis with UV laser-induced fluorescence detection with a He-Cd laser. The separation is carried out within 10 min, and the limit of detection is 2 ng/ml.

Bhatt J[98] ***et al*** developed a method for quantification of zolpidem in human plasma using liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI MS/MS). Es-citalopram was used as an internal standard. Zolpidem and internal standard in plasma sample was extracted using solid-phase extraction cartridge. The samples were injected into a C8 reversed-phase column and the mobile phase used was acetonitrile-ammonium acetate (pH 4.6; 10 mm) (80:20, v/v) at a flow rate of 0.7 ml/min. Using MS/MS in the selected reaction-monitoring (SRM) mode, zolpidem and Es-citalopram were detected without any interference from human plasma matrix. The proposed method was validated in the linear range 2.5-300 ng/ml.

Keller T[99] ***et al*** reported a method for analysing and determining the zolpidem concentration in post-mortem specimens by GC-MS method. Quantitation of zolpidem was performed by ethyl acetate extraction from alkalinized body fluids before GC/MS analysis. The analyses were performed without any complex sample clean-up steps and with little sample material.

Stanke F[100] ***et al*** developed a method for the simultaneous determination of zolpidem and zopiclone in human plasma. After a liquid-liquid extraction, the extract is injected into a capillary gas chromatograph with an OV-1 fused-silica column coupled to a nitrogen-phosphorus detector. The detection limits are 1 and 2 ng/ml for zolpidem and zopiclone, respectively.

Debruyne. D[101] ***et al*** developed a gas chromatographic method using a short, high-resolution capillary column connected to a specific thermionic detector and requiring a simple and short extraction step without evaporation was developed for the rapid and precise determination of zolpidem and zopiclone in serum. The linearity range was between 5 and 200 ng/ml. The method was validated and then used to analyze zolpidem serum concentrations in nine rabbits after oral administration of 0.5 mg/kg and zopiclone serum concentrations in six patients treated orally with a 7.5-g dose.

Nirogi RVS[102] ***et al*** developed a HPLC method with fluorescence detection for quantitation of zolpidem in human plasma. Following a single-step liquid–liquid extraction, the analyte and internal standard (quinine) were separated using an isocratic mobile phase on a reversed-phase C_{18} column. The linearity range of the method was 1.8–288 ng/ml.

Laviana L[103] ***et al*** reported a HPLC assay with diode-array detection for the in-process control of zolpidem synthesis and for the analysis of the drug and its synthetic intermediates. The separation was achieved by reversed-phase Kromasil C-18 (150 mm) column. Acetonitrile and 0.02 M NH_4OAc adjusted to pH 8.0 was used as mobile phase with flow rate 1.0 ml/min.

Ptacek P[104] ***et al*** developed a method for the determination of zolpidem in human plasma by HPLC. This method involves protein precipitation with methanol and reversed-phase chromatography with fluorescence detection (excitation wavelength 244 nm, emission wavelength 388 nm). Methanol- 30 mM dihydrogen potassium phosphate-triethylamine are

used as the mobile phase in the ratio of 30:69:1 and the calibration curve is linear up to 400 ng/ml.

Qiao Wang[105] ***et al*** a single-solvent extraction step high-performance liquid chromatographic method for quantitation of zolpidem in rat serum. The separation was achieved by 2.1 mm I.D. reversed-phase OD-5-100 C_{18} column, 5 μm particle size with an isocratic mobile phase consisting of methanol–acetonitrile– tetrabutyl ammonium phosphate (13:10:77, v/v/v). Ultraviolet detector was operated at 240 nm. The recovery was greater than 87% with analysis performed in 12 min.

El Zeany BA[106] ***et al*** reported two methods for the determination of zolpidem hemitartrate in presence of its degradation product. The first method was a TLC-UV densitometric one in which the mobile phase methanol: water (20:80) was used for developing the TLC plates. Linearity range was 0.5-4 microg/spot with mean recovery percentage (99.98+/-0.988) %. The second method was an HPLC method. HPLC was performed on a bondapack C_{18} column. The mobile phase was composed of a mixture of acetonitrile-0.01 M KH_2PO_4 (40:60). The pH was adjusted to 3.5+/-0.1. Flow rate was 1.2 ml/min. The linearity range was 0.5-5 μg/ml with UV detection at 245 nm.

Ring PR[107]**, *et al*** developed a HPLC method for the determination of zolpidem in human plasma. Zolpidem and the trazodone (internal standard) were extracted from human plasma. Separation was achieved on a C18 column (150 x 4.6 mm, 5microm) with a mobile phase composed of acetonitrile: 50 mM potassium phosphate monobasic at pH 6.0 (4:6 v/v) Detection was by fluorescence, with excitation at 254 nm and emission at 400 nm. The linearity range 1-400 ng/ml for zolpidem in human plasma.

Rajiv Chomwal[108] ***et al*** developed four spectrophotometric methods for estimation of Zolpidem tartrate in tablet dosage forms. The four methods were developed using dyes, bromo phenol red, bromo cresol purple, bromo cresol green and bromo phenol blue

respectively. The linearity ranges of four methods were 5-30 µg/ml, 5-30 µg/ml, 10-50 µg/ml and 5-40 µg/ml respectively. Absorbance maxima measured at 407 nm, 417 nm, 412 nm and 415 nm respectively.

Patil KS[109] ***et al*** reported a spectrophotometric method for estimation of Zolpidem tartrate in bulk and pharmaceutical dosage forms. Zolpidem tartrate shows maximum absorbance at 238.5. Beer's law was obeyed in the concentration range of 2-16 µg/ml. The limit of detection and limit of quantification were found to be 0.038152 µg/ml and 0.114577 µg/ml respectively.

REAGENTS

NQS REAGENT

Mahmoud AM[110] ***et al*** developed a spectrophotometric and spectrofluorimetric method for the determination of amantadine hydrochloride (AMD) in capsules and plasma. The methods were based on the condensation of AMD with 1, 2-naphthoquinone-4-sulphonate (NQS) in an alkaline medium to form an orange-colored product. The spectrophotometric method involved the measurement of the colored product at 460 nm. The spectrofluorimetric method involved the reduction of the product with potassium borohydride, and the subsequent measurement of the formed fluorescent reduced AMD-NQS product at 382 nm after excitation at 293 nm. Linearity ranges were 5–80 and 0.05–10 µg/ml for the spectrophotometric and spectrofluorimetric methods, respectively.

Huai You Wang[111] ***et al*** developed a spectrophotometric method for determination of dapsone. The dapsone reacts with sodium 1, 2-naphthoquinone-4-sulfonic in pH 6.98 buffer solution to form a salmon pink compound, and its maximum absorption wavelength is at 525 nm. The linearity range was 0.40 to 10 µg/ml.

SANGERS REAGENT

Abdel Razak O[112] ***et al*** developed a method for spectrophotometric and polarographic determination of enalapril and lisinopril using 2, 4-dinitrofluorobenzene. The reaction of enalapril maleate and lisinopril with 2,4-dinitrofluorobenzene has been used to form colored products and polarographically active derivatives. The different experimental conditions have been optimized.

MBTH REAGENT AND PARACHLORANILIC ACID

Alfaraj NA[113] ***et al*** developed spectrophotometric methods for the determination of mefenamic acid. The first method is based on the reaction of mefenamic acid as N-donor with *p*-chloranilic acid as a π-acceptor. A red colour product shows peak at 520 nm and its absorbance is linear with concentration over the range 10-300 μg/mL. The second method is based on the formation of an oxidative coupling product by the reaction of mefenamic acid with 3-methylbenzo-thiazolin-2-one hydrazone as a chromogenic reagent in presence of ferric chloride solution. A green colour product shows peak at 602 nm and its absorbance is linear with concentration over the range 1-6 μg/ml.

Manish majumder[114] ***et al*** developed a visible spectrophotometric method for the estimation of tinofovir in bulk and in pharmaceutical preparations. Tinofovir was subjected to acid hydrolysis and this acid hydrolyzed drug was used for the estimation. This method is based on the reaction with 3-methyl-2-benzothiazolinone hydrazone in the presence of ferric chloride, to form a colored species with a λmax at 628.5 nm. Beer's law is obeyed in the concentration range of 5-40 μg/ml.

GIBB'S REAGENT

Chilukuri sastry SP[115] ***et al*** reported two visible spectrophotometric methods for the assay of cefadroxil. First method was based on the reaction of cefadroxil with 3-methyl-2-benzothiazolinone hydrazone hydrochloride in the presence of ceric ammonium sulphate and

the formed colored species was detected at 410 nm. Second method based on reaction of cefadroxil with 2,6-dichloroquinone-4-chlorimideand the formed colored species was detected at 620 nm. Beer's law was obeyed in the concentration range of 1.0-6.0 and 1.0-6.0 μg/ml for first and second methods respectively.

BM REAGENT

Gurupadayya BM[116] ***et al*** developed a method for the quantitative estimation of pramipexole dihydrochloride drug and its formulations (Tablets). This method was based on the diazotization of primary amine group of pramipexole with sodium nitrate and hydrochloric acid followed by coupling with N-(1-napthyl) ethylene diamine hydrochloride (BM reagent) to form a colored chromogen with a characteristic absorption maximum at 616 nm. Beer's law is obeyed in concentrations ranging of 4-20 μg/ml.

2,4-DNP REAGENT

Padmarajaiah Nagaraja[117] ***et al*** developed a spectrophotometric method for the determination of four phenolic drugs; salbutamol, ritodrine, amoxicillin and isoxsuprine. The method is based on the oxidation of 2, 4- Dinitrophenylhydrazine and coupling of the oxidized product with drugs to give intensely colored chromogen. Under the proposed optimum condition, Beer's law was obeyed in the concentration range of 2.5-17, 2-29, 4-33 and 5-30 μg/ml for salbutamol, ritodrine, amoxicillin and isoxsuprine respectively.

OBJECTIVES

The primary objective of the proposed work was to

- Develop new, simple, sensitive, accurate, and economical spectrophotometric methods for the estimation of CNS acting drugs.
- Develop new, sensitive, accurate and economical gas chromatography methods for estimation of CNS acting drugs.
- Validate the developed methods and apply them for estimation of commercially available formulations.

Pregabalin and zolpidem are the drugs belonging to CNS category which are used against epilepsy and insomnia respectively. Pregabalin is an anticonvulsant drug used for neuropathic pain and as an adjunct therapy for partial seizures with or without secondary generalization in adults. Zolpidem is a prescription medication used for the short-term treatment of insomnia, as well as some brain disorders. It is a short-acting nonbenzodiazepine

hypnotic that potentiates gamma-amino butyric acid (GABA), an inhibitory neurotransmitter by binding to $GABA_A$ receptors at the same location as benzodiazepines.

Literature survey reveals that very few methods have been developed for pregabalin and zolpidem. In the present investigation made attempts to develop some new spectrophotometric methods and chromatographic methods for pregabalin and zolpidem, which are highly sensitive, accurate, precise and economical. Since very few analytical methods have been reported for the quantitative estimation of these drugs its necessary for the investigation of new analytical methods for estimation of pregabalin and zolpidem in bulk drugs and pharmaceutical formulations.

Validation of the methods was carried out in accordance with ICH guideline for the assay of active ingredients. The method was validated for parameters like accuracy, linearity, precision, specificity, system suitability.

The primary objective of validation in the analysis of a drug is to design and develop methods preferably instrumental ones such as UV spectrophotometric / GC that are sensitive and reproducible, when applied for analysis of marketed formulations.

MATERIALS AND METHODS

PART A:

UV-VISIBLE SPECTROPHOTOMETRIC METHODS FOR PREGABALIN

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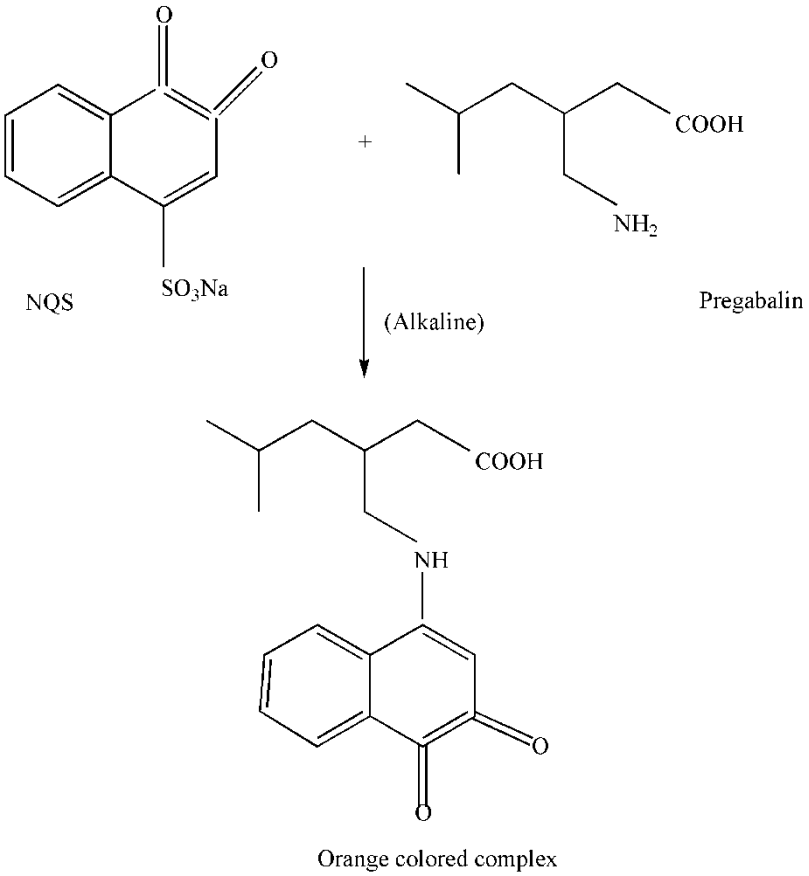
PART A: UV- VISIBLE SPECTROPHOTOMERTIC METHODS

METHOD 1 (ESTIMATION OF PREGABALIN BY NQS REAGENT)

1.1 PRINCIPLE INVOLVED

Pregabalin possesses different functional groups such as carboxylic acid and free amine group. An attempt has been made to determine pregabalin by reacting the amine group with the reagent. The reaction involves replacement of SO_3Na group in NQS by the amino group in pregabalin in alkaline condition which is as shown below **(Scheme 1)**. The reaction results in an orange coloured complex with absorption maximum at 485 nm.

1.2 REACTION INVOLVED



Scheme 1: Reaction of NQS reagent and PGB

1.3 REAGENTS USED

1.3.1 Sodium Hydroxide 0.01N

0.2 g of sodium hydroxide was weighed accurately and dissolved in distilled water in 500 ml volumetric flask and volume was made up to mark with distilled water.

1.3.2 NQS 0.5 % (w/v)

0.50 g of NQS was weighed accurately and dissolved in distilled water in 100 ml volumetric flask and volume was made up to mark with distilled water.

1.4 PREPARATION OF STANDARD CALIBRATION CURVE

1.4.1 Preparation of standard stock solution

Accurately weighed 100 mg of pregabalin (bulk drug) was dissolved in 40 ml of warm double distilled water in 100 ml volumetric flask and sonicated for about 15 min to enhance the solubility and volume was made up to the mark with double distilled water to obtain a final concentration of 1000 µg/ml (solution A).

From the above stock solution A 10 ml of aliquot was pipetted out in 100 ml volumetric flask and the volume was made up to the mark with double distilled water to obtain the final concentration of 100 µg/ml (Stock solution B).

1.4.2 Preparation of calibration curve

Aliquots of pregabalin ranging from 0.5- 4.5 ml (5- 45µg/ml) were pipetted into a series of 10 ml volumetric flask. To each flask, 1.0 ml of NQS solution and 1.0 ml of 0.01N sodium hydroxide were added and diluted to volume with distilled water. The reaction was allowed to proceed at room temperature. The absorption spectrum of PGB was done and it showed 485 nm as the maximum absorption point (Fig.1). The calibration curve was constructed by plotting absorbance against the concentration of PGB. The linearity range or Beer's range follows in the range between 5- 45 µg/ml (Fig.2). The content of PGB was calculated from the calibration graph.

1.5 ANALYSIS OF CAPSULE DOSAGE FORM

Five capsules (Pregeb – 75mg) were weighed and their contents were mixed thoroughly. An accurately weighed portion of powder equivalent to the 100 mg of PGB was weighed into a 100 ml volumetric flask containing about 75 ml of warm distilled water. It was shaken thoroughly for about 5-10 min, filtered thorough whatman filter paper to remove insoluble matter and diluted to the mark with distilled water to 1000 µg/ml solution (stock solution A). From the above stock solution 10.0 ml was pipetted out in 100 ml volumetric flask and the volume was made up with warm double distilled water to obtain final concentration of 100 µg/ml. From stock solution B 4.0 ml of solution was pipetted into a 10 ml volumetric flask and then resultant solution is also analysed as per the procedure and were statistically validated.

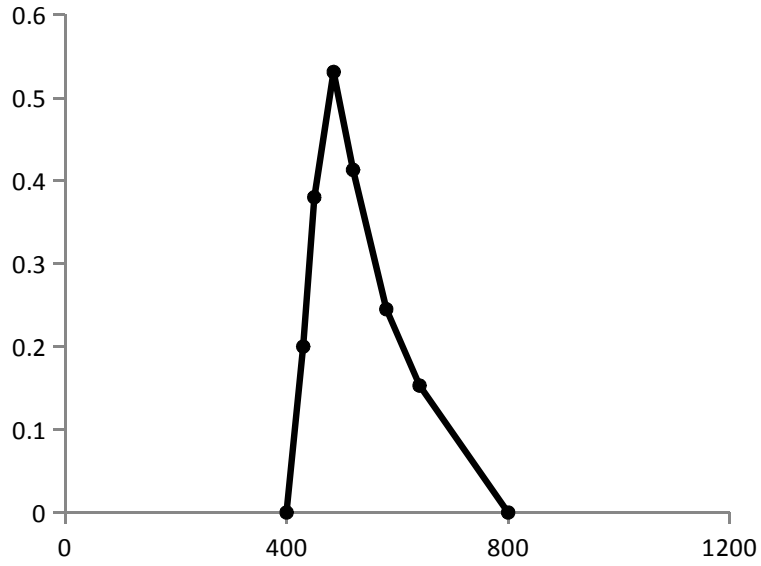


Fig.1. Absorption spectra of NQS with PGB against the reagent blank

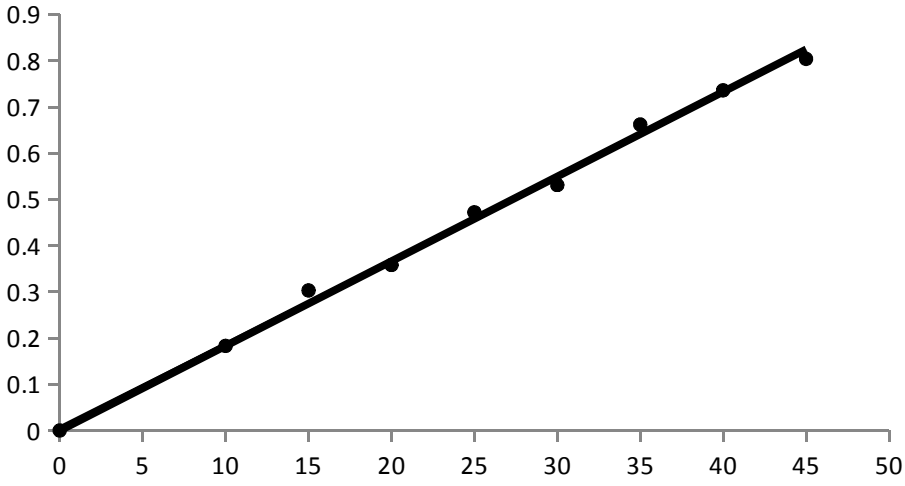


Fig.2. Calibration graph of PGB with NQS reagent

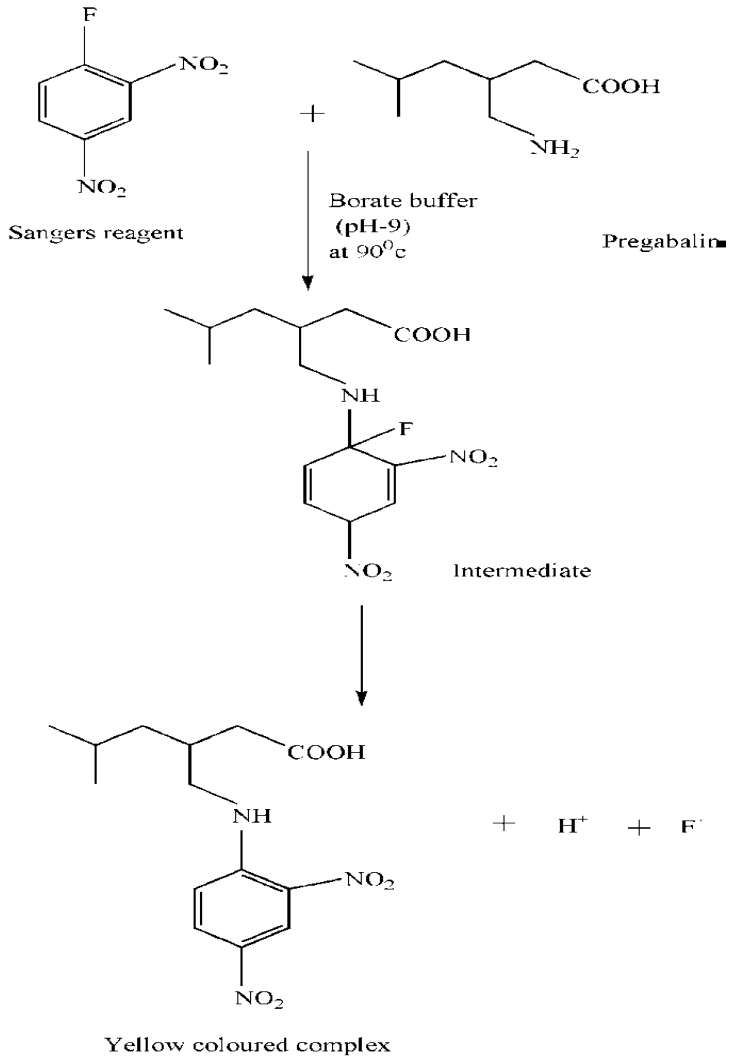
METHOD 2 (ESTIMATION OF PREGABALIN BY SANGERS REAGENT)

2.1 PRINCIPLE INVOLVED

In this method an attempt has been made to determine pregabalin by reacting the amine group with sangers reagent. The reaction involves replacement of fluorine in sangers reagent by the amine group in pregabalin in alkaline condition which is as shown below

(Scheme 2). The reaction results in a yellow coloured complex with absorption maximum at 353 nm.

2.2 REACTION INVOLVED



Scheme 2: Reaction of sangers reagent and PGB

2.3 REAGENTS USED

2.3.1 Sanger's reagent 0.5 %(w/v)

0.5 g of Sanger's reagent was accurately weighed and transferred into a 100 ml calibrated flask, dissolved in methanol, and make up the volume up to the mark to obtain a

solution of 0.5% (w/v). Reagent should be protected from light during use and should be handled carefully since it is a skin irritant. If stored in a refrigerator it is stable for 4 months.

2.3.2 Borate buffer pH 9

Placed 50 ml of the 0.2 M boric acid and potassium chloride in 200 ml volumetric flask, added 20.0 ml of 0.2 M NaOH and made to the mark with water.

2.4 PREPARATION OF STANDARD CALIBRATION CURVE

2.4.1 Preparation of standard stock solution

Accurately weighed 100.0 mg of pregabalin (bulk drug) was dissolved in 40.0 ml of methanol in 100 ml volumetric flask and sonicated for about 15 min to enhance the solubility and volume was made up to the mark with methanol to obtain a final concentration of 1000 µg/ml (solution A).

From the above stock solution A 10 ml of aliquot was pipetted out in 100 ml volumetric flask and the volume was made up to the mark with methanol to obtain the final concentration of 100 µg/ml (solution B).

2.4.2 Preparation of calibration curve

Aliquots of pregabalin ranging from 0.5- 1.3 ml (1.0 - 13.0 µg/ml) were pipetted into as series of 10 ml volumetric flask. To each flask, 1.0 ml of sanger's solution and 0.2 ml of borate buffer pH 9 were added. The mixture was then gently shaken and heated for 90^0C for 10 minutes until the appearance of yellow colour. The contents were diluted up to 10 ml with methanol. The absorption spectrum of PGB was done and it showed 353 nm as the maximum absorption point (Fig.3). The calibration curve was constructed by plotting absorbance against the initial concentration of PGB. The linearity range or Beer's range follows in the range between 5.0- 13.0 µg/ml (Fig.4). The content of PGB was calculated from the calibration graph.

2.5 ANALYSIS OF CAPSULE DOSAGE FORM

Five capsules were weighed and their contents were mixed thoroughly. An accurately weighed portion of powder equivalent to the 100 mg of PGB was weighed into a 100 ml

volumetric flask containing about 50 ml of methanol. It was shaken thoroughly for about 5-10 min, filtered through whatman filter paper to remove insoluble matter and diluted to the mark with methanol to prepare 1000 µg/ml solution (stock solution A). From the above stock solution 10.0 ml was pipetted out in 100 ml volumetric flask and the volume was made up with methanol to obtain final concentration of 100 µg/ml. From the above stock pipetted out 1 ml in 10 ml volumetric flask and the resultant solution was analysed as per the procedure and were statistically validated.

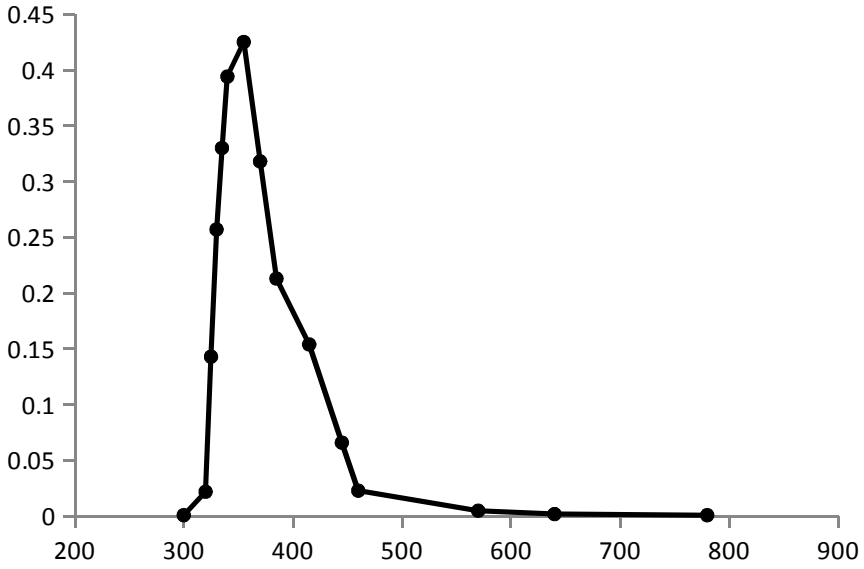


Fig.3. Absorption spectra of sangers with PGB against the reagent blank

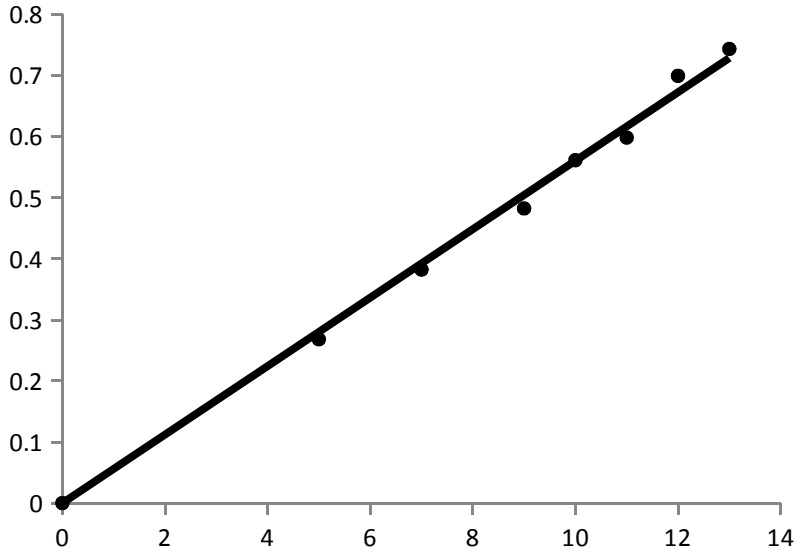


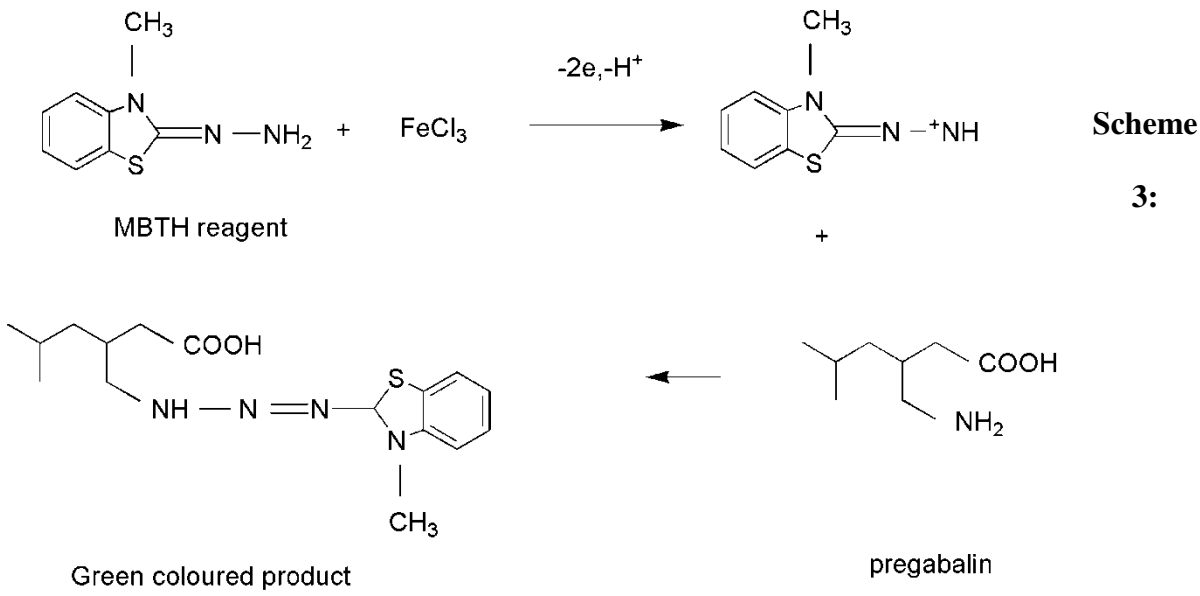
Fig.4. Calibration graph of PGB with Sangers reagent

METHOD 3 (ESTIMATION OF PREGABALIN BY MBTH REAGENT)

3.1 PRINCIPLE INVOLVED

In this method PGB reacts with MBTH in the presence of $FeCl_3$ to give a green colored product **(Scheme 3)**. This is an iron catalyzed oxidative coupling reaction of MBTH with the drug. Under the reaction conditions, on oxidation, MBTH loses two electrons and one proton forming an electrophilic intermediate, which is the active coupling species. This intermediate undergoes electrophilic substitution with the drug to form green colored product with absorption maximum of 668 nm.

3.2 REACTION INVOLVED



Scheme 3:

3.3 REAGENTS USED

3.3.1 3-Methyl-2-Benzthiazolinone hydrochloride (MBTH) 0.5 %(w/v)

0.5 g of MBTH reagent was accurately weighed transferred into a 100 ml calibrated flask, dissolved in distilled water and made to the mark to obtain a solution of 0.5% (w/v).

3.3.2 Ferric chloride (1%)

It was prepared by dissolving 1.0 g of ferric chloride in 100 ml of distilled water.

3.4 PREPARATION OF STANDARD CALIBRATION CURVE

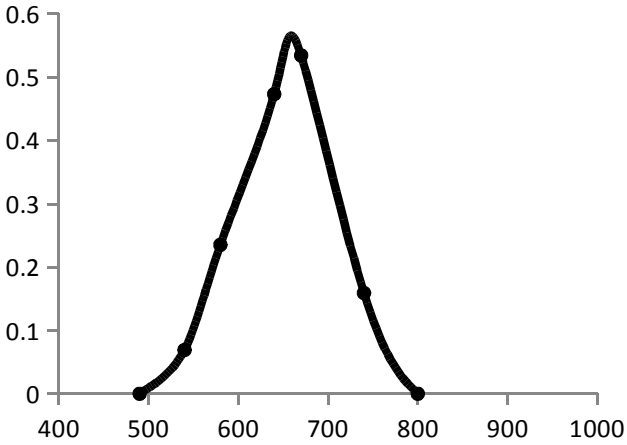
3.4.1 Preparation of standard stock solution

Accurately weighed 100.0 mg of pregabalin (bulk drug) was dissolved in 40.0 ml of methanol in 100 ml volumetric flask and sonicated for about 15 min to enhance the solubility and volume was made up to the mark with methanol to obtain final concentration of 1.0 mg/ml.

3.4.2 Preparation of calibration curve

Aliquots of pregabalin ranging from 0.5- 3.5 ml (50- 350 µg/ml) were pipetted into a series of 10 ml volumetric flask. To each flask 2 ml of MBTH, 2 ml of ferric chloride was added and the volume was made to the mark with distilled water and allowed to stand for 20 minutes. The absorbance of each solution was measured at 668 nm against the reagent blank. The colored species was stable for 2 hours and the amount of drug in the sample was computed from its calibration curve represented in Fig. 5. and Fig. 6.

3.5 ANALYSIS OF CAPSULE DOSAGE FORM



Five capsules were weighed and their contents are mixed thoroughly. An accurately weighed portion of powder equivalent to the 100 mg of PGB was weighed into a 100 ml volumetric flask containing about 50 ml of methanol. It was shaken thoroughly for about 5-10 minutes, filtered through a whatman filter paper to remove insoluble matter and diluted to the mark with methanol to prepare 1000 µg/ml solution. 1.0 ml of the above solution was taken from above stock solution in to a 10 ml volumetric flask. Then to that solution 2 ml of MBTH and 2 ml of $FeCl_3$ were added. The mixture was then gently shaken and the appearance of green color occurs. The contents were diluted up to 10 ml with distilled water.

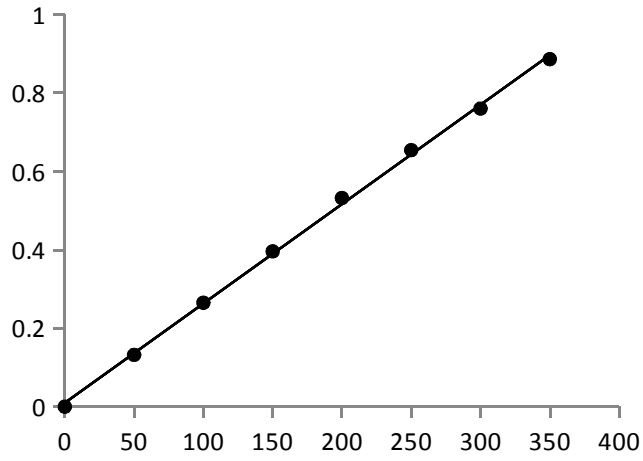


Fig.5. Absorption spectra of MBTH with PGB against the reagent blank

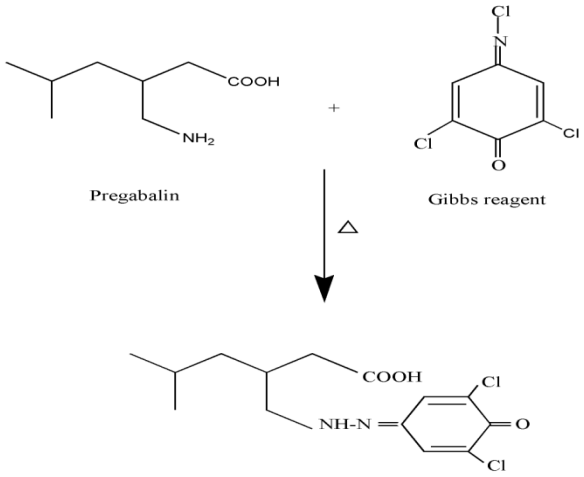
Fig.6. Calibration graph of PGB with MBTH reagent

METHOD 4 (ESTIMATION OF PREGABALIN BY GIBB'S REAGENT)

4.1 PRINCIPLE INVOLVED

In this method an attempt has been made to determine pregabalin by reacting the amine group with Gibb's reagent. The reaction involves oxidation of the pregabalin with reagent **(Scheme 4)**. The colored product had absorbance maxima at 400 nm.

4.2 REACTION INVOLVED



Scheme 4: Reaction of Gibb's reagent and PGB

4.3 REAGENTS USED

4.3.1 2, 6-Dichloroquinone-chlorimide 0.5 %(w/v):

0.5 g of Gibb's reagent was accurately weighed transferred into a 100 ml calibrated flask, dissolved in 10 ml of methanol, and made to the mark with methanol to obtain a solution of 0.5% (w/v). The solution was freshly prepared and protected from light.

4.4 PREPARATION OF STANDARD CALIBRATION CURVE

4.4.1 Preparation of standard stock solution

Accurately weighed 100.0 mg of pregabalin (bulk drug) was dissolved in 40.0 ml of methanol in 100 ml volumetric flask and sonicated for about 15 min to enhance the solubility and volume was made up to the mark with methanol to obtain final concentration of 1.0 mg/ml.

4.4.2 Preparation of calibration curve

Aliquots of pregabalin ranging from 0.5- 3.5 ml (50- 350 µg/ml) were pipetted into a series of 10 ml volumetric flask. To those solutions 1.5 ml of 0.5% Gibb's reagent was added. The mixture was then kept aside for 5 minutes and heated for 10 minutes. The contents were diluted to 10 ml with methanol. The absorbance of each solution was measured at 400 nm

against the reagent blank. The colored species was stable for 2 hours and the amount of drug in the sample was computed from its calibration curve represented in Fig. 7 and Fig. 8.

4.5 ANALYSIS OF CAPSULE DOSAGE FORM

Five capsules were weighed and their contents are mixed thoroughly. An accurately weighed portion of powder equivalent to the 100 mg of PGB was weighed into a 100 ml volumetric flask containing about 75 ml of methanol. It was shaken thoroughly for about 5-10 minutes, filtered through a whatman filter paper to remove insoluble matter and diluted to the mark with methanol to prepare 1000 µg/ml solution. An aliquot of this solution was diluted with methanol to obtain a concentration of 50 µg/ml. Then to that solution, 1 ml of Gibb's reagent was added and then gently shaken. The contents were diluted up to 10 ml with methanol.

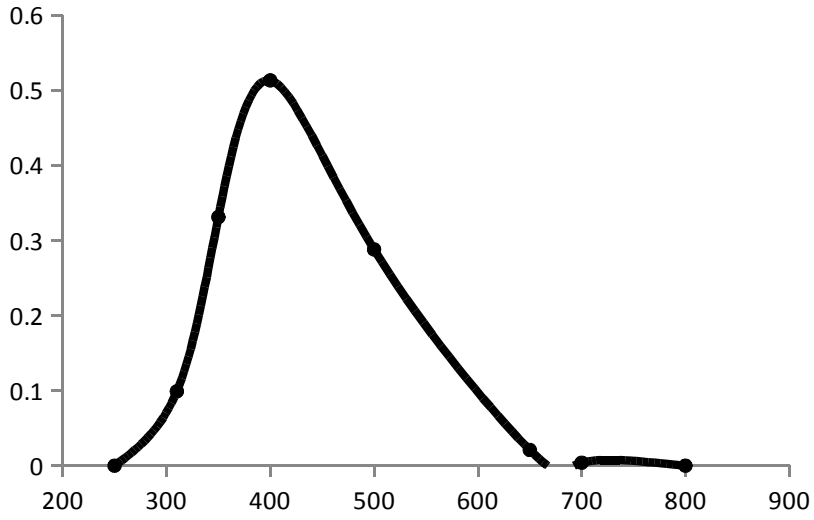


Fig.7. Absorption spectra of Gibb's with PGB against the reagent blank

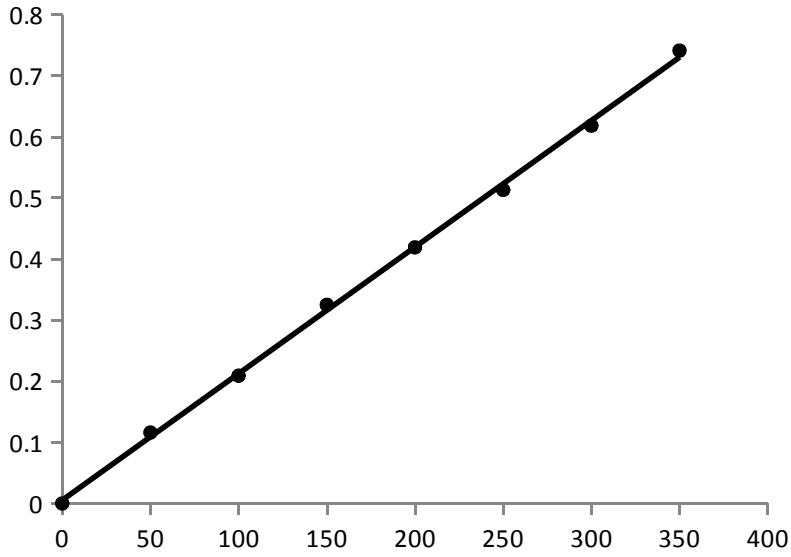


Fig.8. Calibration graph of PGB with Gibb's reagent

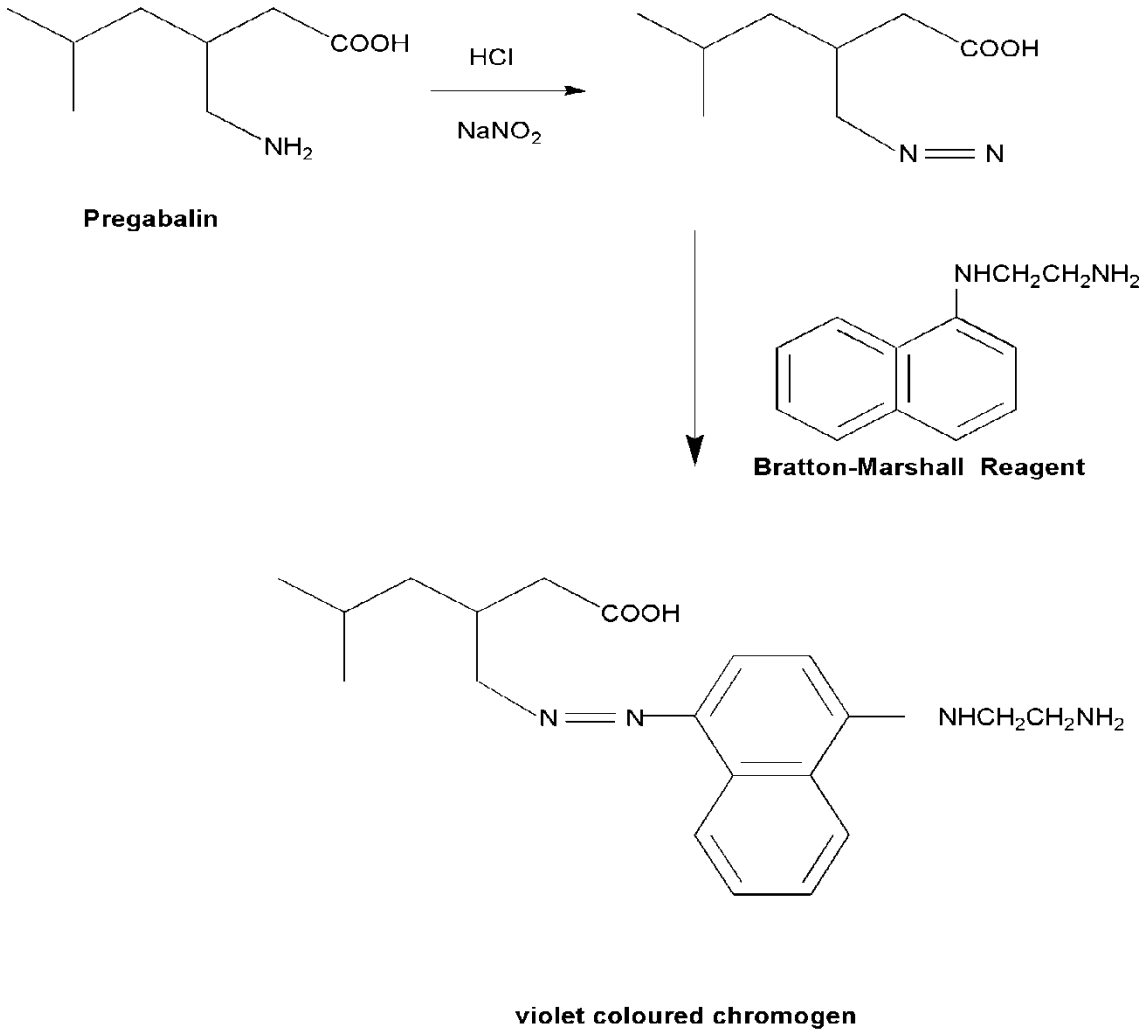
METHOD 5 (ESTIMATION OF PREGABALIN BY BM REAGENT)

5.1 PRINCIPLE INVOLVED

In this method the reaction between pregabalin and BM reagent depends on diazotization of drugs with nitrous acid, to form diazotized compound, followed by its

coupling with N-(1-naphthyl) ethylene- diamine dihydrochloride [Bratton-Marshal reagent] to form a violet colored chromogen **(Scheme 5)** with absorption maximum of 471 nm.

5.2 REACTION INVOLVED



Pregabalin

Bratton-Marshall Reagent

violet coloured chromogen

5.3 REAGENTS USED

5.3.1 2N HCl:

It was prepared by dissolving 8.5 ml of hydrochloric acid in 100 ml of water.

5.3.2 Sodium nitrite 0.3 % (w/v):

A 0.3% (w/v) sodium nitrite was prepared by dissolving 0.3 g in 100 ml of distilled water.

5.3.3 Ammonium sulfamate 0.1 % (w/v):

A 0.1 % (w/v) ammonium sulfamate was prepared by dissolving 0.1 g in 100 ml of distilled water.

5.3.4 N-(1-naphthyl) ethylene- diamine dihydrochloride [BMR] 0.2 % (w/v):

A 0.2 % (w/v) BM reagent was prepared by dissolving 0.2 g in 100 ml of distilled water.

5.4 PREPARATION OF STANDARD CALIBRATION CURVE

5.4.1 Preparation of standard stock solution

Accurately weighed 100.0 mg of pregabalin (bulk drug) was dissolved in 40.0 ml of methanol in 100 ml volumetric flask and sonicated for about 15 min to enhance the solubility and volume was made up to the mark with methanol to obtain final concentration of 1.0 mg/ml.

5.4.2 Preparation of calibration curve

Aliquots of pregabalin ranging from 0.5- 3.5 ml (50- 350 µg/ml) were pipetted into a series of 10 ml volumetric flask. To each flask, 1.0 ml of hydrochloric acid (2 N) and 1.0 ml of sodium nitrite (0.3 % w/v) were added and a reaction time of 10 minutes to these solutions at 0-5˚ C was given for the completion of the reaction. Next, 1.0 ml of ammonium sulfamate (0.1 % w/v) was added to each flask with gentle shaking after 1 minute, 1 ml of BMR reagent (0.2 % w/v) was added, and kept for 20 minutes. Finally the volume in each flask was brought up to the 10 ml mark with distilled water. The absorbance of violet-coloured

chromogen was measured at 471 nm. The amount of drug in the sample was computed from its calibration curve represented in Fig. 9 and Fig. 10.

5.5 ANALYSIS O DOSAGE FORM

Five capsules were weighed and their contents are mixed thoroughly. An accurately weighed portion of powder equivalent to the 100 mg of PGB was weighed into a 100 ml volumetric flask containing about 75 ml of methanol. It was shaken thoroughly for about 5-10 minutes, filtered through a whatman filter paper to remove insoluble matter and diluted to the mark with methanol to prepare 1000 µg/ml solution. From the above stock solution 0.5 ml was taken in a 10 ml volumetric flask and the resultant solution was analysed as per the procedure and were statistically validated.

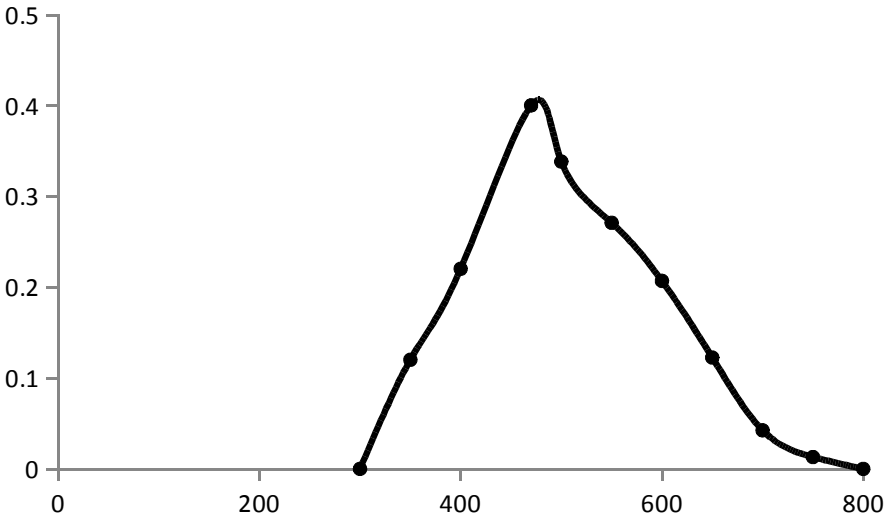


Fig .9. Absorption spectra of BM reagent with PGB against the reagent blank

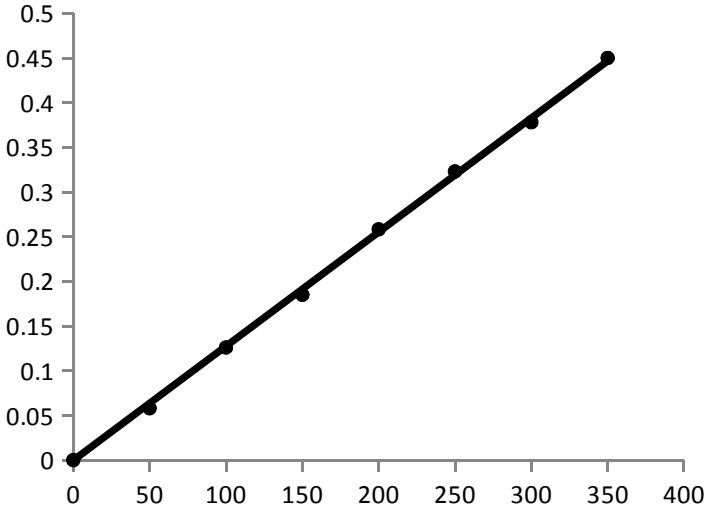


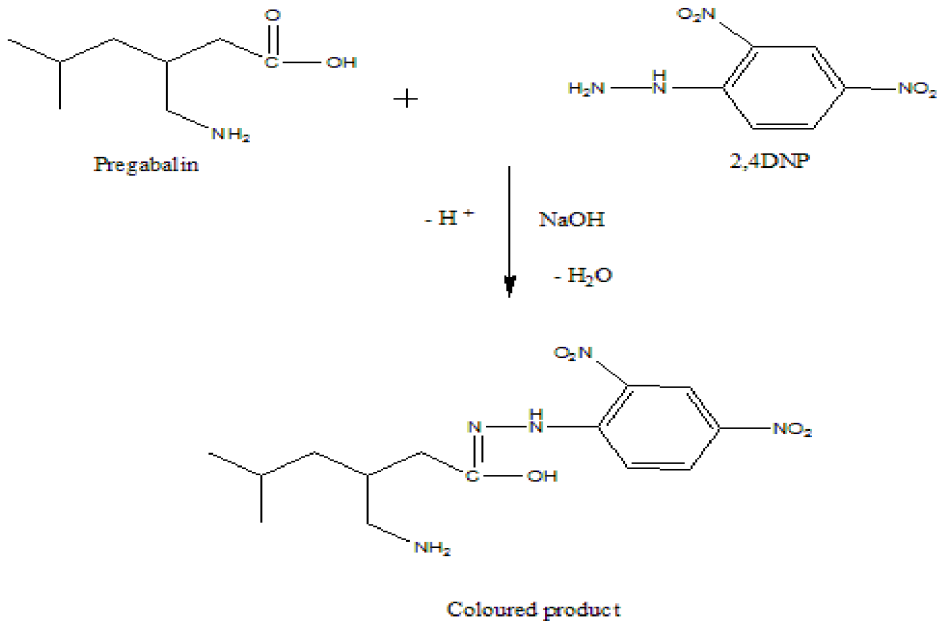
Fig .10. Calibration graph of PGB with BM reagent

METHOD 6 (ESTIMATION OF PREGABALIN BY 2, 4 – DNP REAGENT)

6.1 PRINCIPLE INVOLVED

In this method 2, 4-DNP is oxidized by potassium iodate to give diazonium cation which reacts with carboxylic group of the drug by electrophilic substitution to give deep colored chromogen **(Scheme 6)** with absorption maximum of 461 nm.

6.2 REACTION INVOLVED



Scheme 6: Reaction of 2,4-DNP reagent and PGB

6.3 REAGENTS USED

6.3.1 2, 4-Dinitrophenyl hydrazine (2, 4-DNP) 0.08 %(w/v)

A 0.08% w/v of the reagent solution was freshly prepared by dissolving 0.08 g of 2, 4-DNP in 2 ml of concentrated sulphuric acid and diluting to 100 ml with water.

6.3.2 10N Sodium hydroxide solution.

40 g of sodium hydroxide was dissolved in 100 ml of distilled water.

6.3.3 Potassium iodate 4 % (w/v)

A 4% w/v potassium iodate solution was prepared by dissolving 4 g in 100 ml of distilled water.

6.4 PREPARATION OF STANDARD CALIBRATION CURVE

6.4.1 Preparation of standard stock solution

Accurately weighed 100.0 mg of pregabalin (bulk drug) was dissolved in 40.0 ml of warm double distilled water in 100 ml volumetric flask and sonicated for about 15 min to enhance the solubility and volume was made up to the mark with double distilled water to obtain final concentration of 1000 µg/ml (Stock solution).

6.4.2 Preparation of calibration curve

Aliquots of pregabalin ranging from 0.5- 3.5 ml (50- 350 µg/ml) were pipetted into a series of 10 ml volumetric flask. To these solutions 1.5 ml of 2, 4-DNP and 1.5 ml of potassium iodate were added, which were made alkaline by adding 1 ml each of sodium hydroxide. The red color hence developed was further diluted to the volume with water. The amount of drug in the sample was computed from its calibration curve represented in Fig. 11 and Fig. 12.

6.5 ANALYSIS O DOSAGE FORM

Five capsules were weighed and their contents are mixed thoroughly. An accurately weighed portion of powder equivalent to the 100 mg of PGB was weighed into a 100 ml

volumetric flask containing about 75 ml of distilled water. It was shaken thoroughly for about 5-10 min, filtered through whatman filter paper to remove insoluble matter and diluted to the mark with distilled water to prepare 1000 µg/ml solution. From the above stock solution 1 ml was taken into 10 ml volumetric flask. Then to that solution 1.5 ml of 2, 4-DNP was added, and 1.5 ml of potassium iodate and 1 ml of 10 N sodium hydroxide was added. The mixture was then gently shaken until the appearance of red color. The contents were diluted up to 10 ml with distilled water to obtain 100 µg/ml concentration.

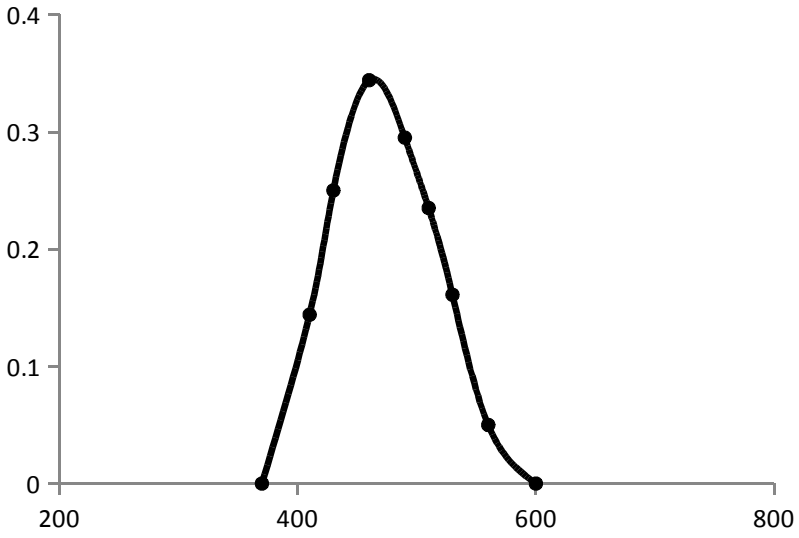


Fig.11. absorption spectra of 2, 4- DNP with PGB against the reagent blank

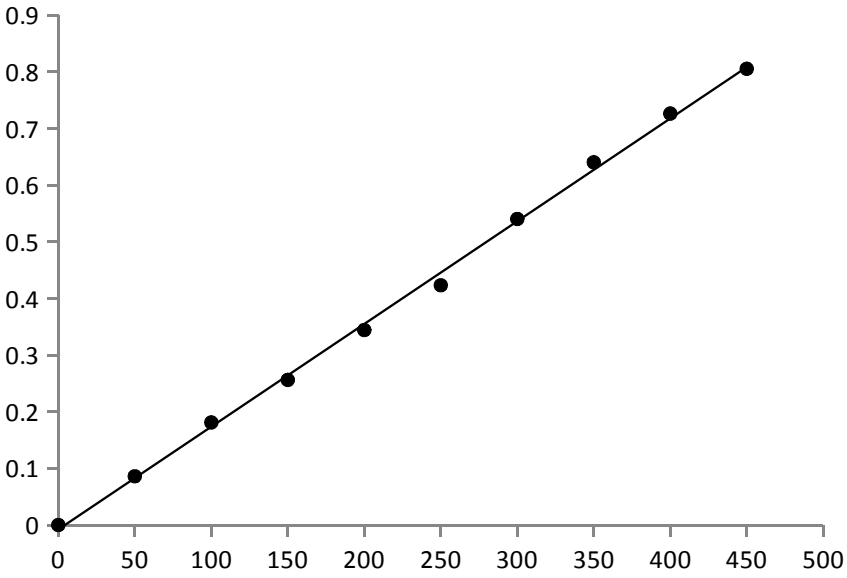


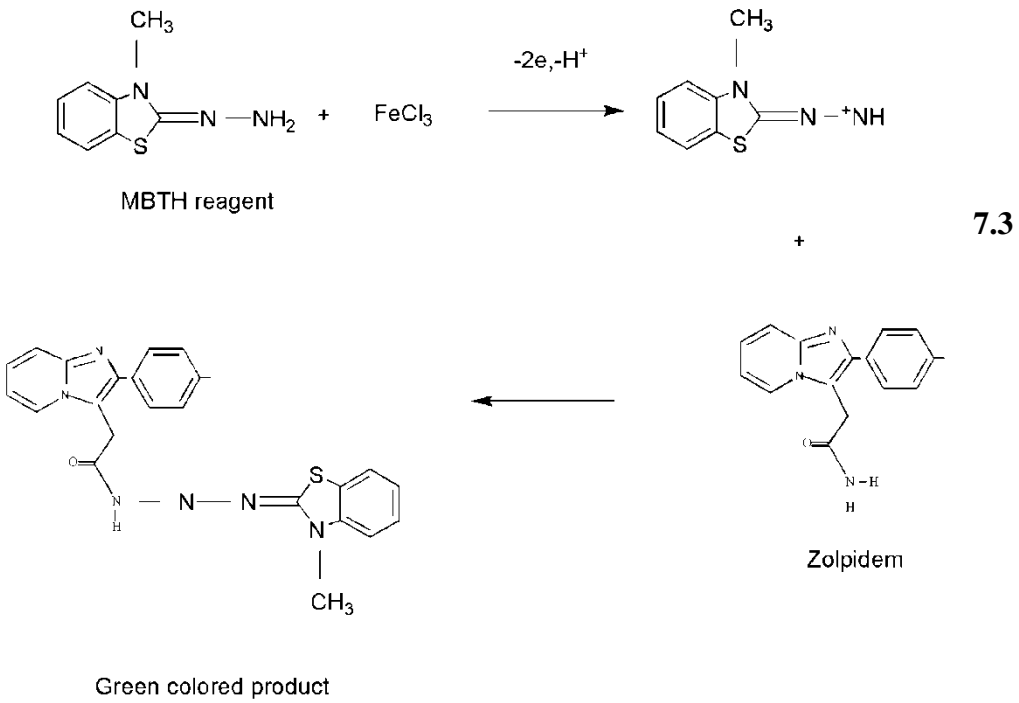
Fig. 12. Calibration graph of PGB with 2, 4-DNP

METHOD 7 (ESTIMATION OF ZOLPIDEM BY MBTH REAGENT)

7.1 PRINCIPLE INVOLVED

Zolpidem contain acetamide (Carbonyl group and amine group) as functional groups. An attempt has been made to determine zolpidem by reacting the secondary amine group with MBTH reagent. The drug reacts with MBTH in the presence of $FeCl_3$ to give a green colored product **(Scheme 7)**. This is an iron catalyzed oxidative coupling reaction of MBTH with the drug. Under the reaction conditions, on oxidation, MBTH loses two electrons and one proton forming an electrophilic intermediate, which is the active coupling species. This intermediate undergoes electrophilic substitution with the drug to form green colored product with absorption maximum of 664 nm.

7.2 REACTION INVOLVED



Scheme 7: Reaction of MBTH reagent and ZT

REAGENTS USED

7.3.1 3-Methyl-2-Benzthiazolinone hydrochloride (MBTH) 0.5 %(w/v)

0.5 g of MBTH reagent was accurately weighed transferred into a 100 ml calibrated flask, dissolved in distilled water, and make up the volume up to the mark to obtain a solution of 0.5% (w/v).

7.3.2 Ferric chloride (1%)

It was prepared by dissolving 1.0 g of ferric chloride in 100 ml of distilled water.

7.4 PREPARATION OF STANDARD CALIBRATION CURVE

7.4.1 Preparation of standard stock solution

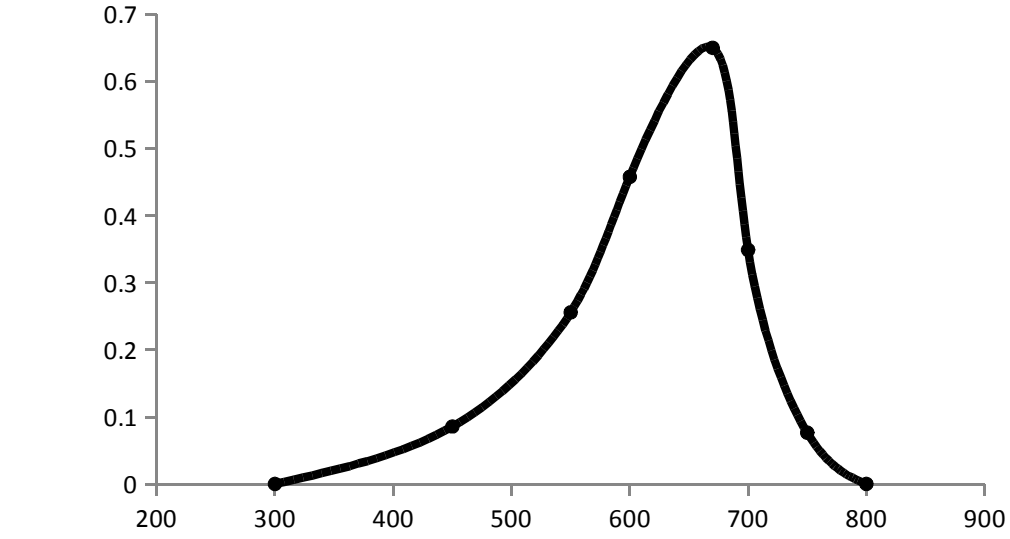
Accurately weighed 100.0 mg of zolpidem (bulk drug) was dissolved in 40.0 ml of methanol in 100 ml volumetric flask and sonicated for about 15 min to enhance the solubility and volume was made up to the mark with methanol to obtain final concentration of 1.0 mg/ml.

7.4.2 Preparation of calibration curve

Aliquots of zolpidem ranging from 0.5- 3.5 ml (50- 350 µg/ml) were pipette into a series of 10 ml volumetric flask. To each flask 2 ml of MBTH, 2 ml of ferric chloride was added and the volume was made up to mark with distilled water and allowed to stand for 20 minutes. The colored species was stable for 2 hours and the amount of drug in the sample was computed from its calibration curve represented in Fig.13 and Fig. 14.

7.5 ANALYSIS OF CAPSULE DOSAGE FORM

10 tablets (Zolfresh 10 mg) were weighed and their contents were mixed thoroughly. An accurately weighed portion of powder equivalent to the 100 mg of zolpidem was weighed into a 100 ml volumetric flask containing about 50 ml of methanol. It was shaken thoroughly for about 5-10 minutes, filtered through whatman filter paper to remove insoluble matter and diluted to the mark with methanol to prepare 1000 µg/ml solution. An aliquot of this solution was diluted with water to obtain a concentration of 100 µg/ml. Then to that solution 2 ml of MBTH and 2 ml of $FeCl_3$ were added. The mixture was then gently shaken and the appearance of green color was observed. The contents were diluted up to 10 ml with distilled water.



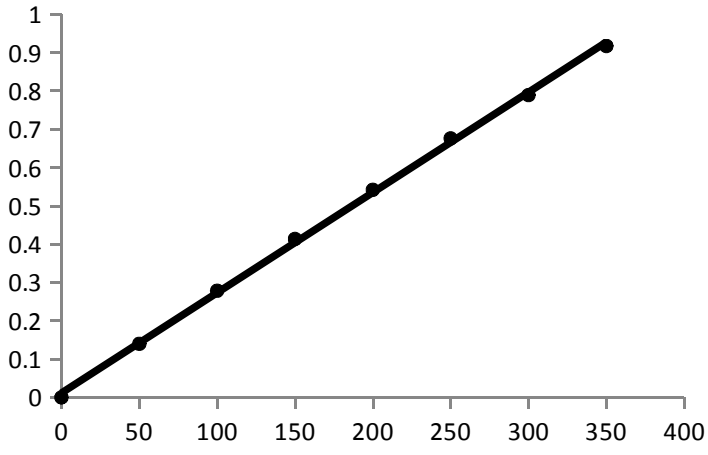


Fig.13. Absorption spectra of MBTH with ZT against the reagent blank

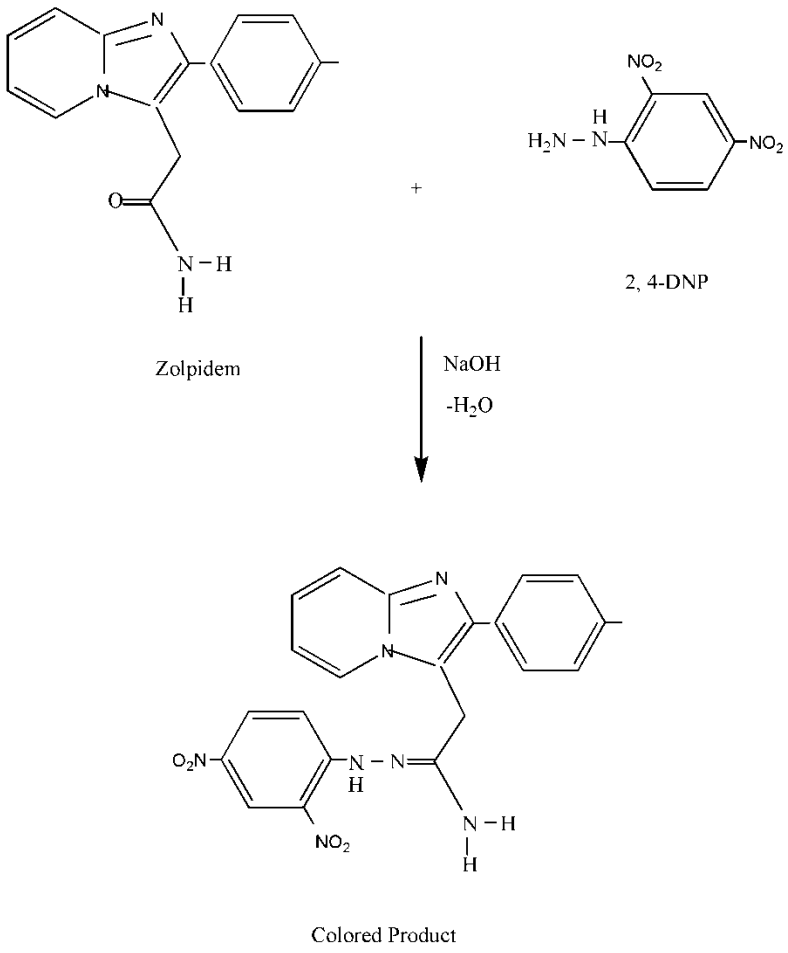
Fig.14. Calibration graph of ZT with MBTH

METHOD 8 (ESTIMATION OF ZOLPIDEM BY 2, 4 – DNP REAGENT)

8.1 PRINCIPLE INVOLVED

In this method 2, 4-DNP is oxidized by potassium iodate to give diazonium cation which reacts with drug by electrophilic substitution to give deep colored chromogen **(Scheme 8)** with absorption maximum of 416 nm.

8.2 REACTION INVOLVED



Scheme 8: Reaction of 2,4-DNP and ZT

8.3 REAGENTS USED

8.3.1 2, 4-Dinitrophenyl hydrazine (2, 4-DNP) 0.08 %(w/v)

A 0.08% w/v of the reagent solution was freshly prepared by dissolving 0.08 g of 2, 4-DNP in 2 ml of concentrated sulphuric acid and diluting to 100 ml with water.

8.3.2 10N Sodium hydroxide solution.

40 g of sodium hydroxide was dissolved in 100 ml of distilled water.

8.3.3 Potassium iodate 4 % (w/v)

A 4% w/v potassium iodate solution was prepared by dissolving 4 g in 100 ml of distilled water.

8.4 PREPARATION OF STANDARD CALIBRATION CURVE

8.4.1 Preparation of standard stock solution

Accurately weighed 100.0 mg of zolpidem (bulk drug) was dissolved in 40.0 ml of methanol in 100 ml volumetric flask and sonicated for about 15 min to enhance the solubility and volume was made up to the mark with methanol to obtain final concentration of 1000 µg/ml (Stock solution).

8.4.2 Preparation of calibration curve

Aliquots of zolpidem ranging from 0.2- 0.9 ml (20- 90 µg/ml) were pipette into a series of 10 ml volumetric flask. To these solutions 1.5 ml of 2, 4-DNP and 1.5 ml of potassium iodate were added, which were made alkaline by adding 1 ml each of sodium hydroxide. The red color hence developed was further diluted to the volume with water. The amount of drug in the sample was computed from its calibration curve represented in Fig. 15 and Fig. 16.

8.5 ANALYSIS O DOSAGE FORM

10 tablets were weighed and their contents were mixed thoroughly. An accurately weighed portion of powder equivalent to the 100 mg of ZT was weighed into a 100 ml volumetric flask containing about 75 ml of methanol. It was shaken thoroughly for about 5-10 min, filtered through whatman filter paper to remove insoluble matter and diluted to the mark with methanol to prepare 1000 µg/ml solution. An aliquot of this solution was taken in a 10 ml volumetric flask to obtain a concentration of 60 µg/ml. Then to that solution 1.5 ml of 2, 4-DNP was added, and 1.5 ml of potassium iodate and 1 ml of 10 N sodium hydroxide

were added. The mixture was then gently shaken until the appearance of red color. The contents were diluted up to 10 ml with methanol.

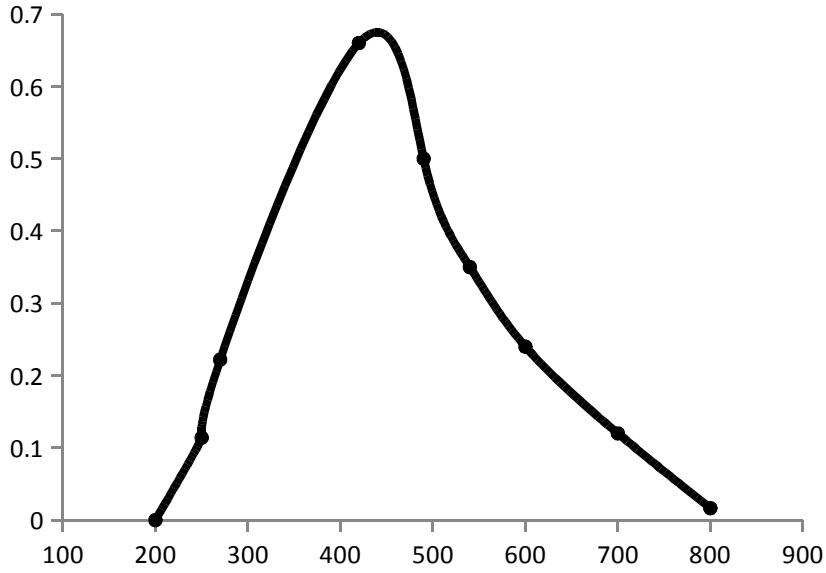


Fig.15. Absorption spectra of 2, 4-DNP with ZT against the reagent blank

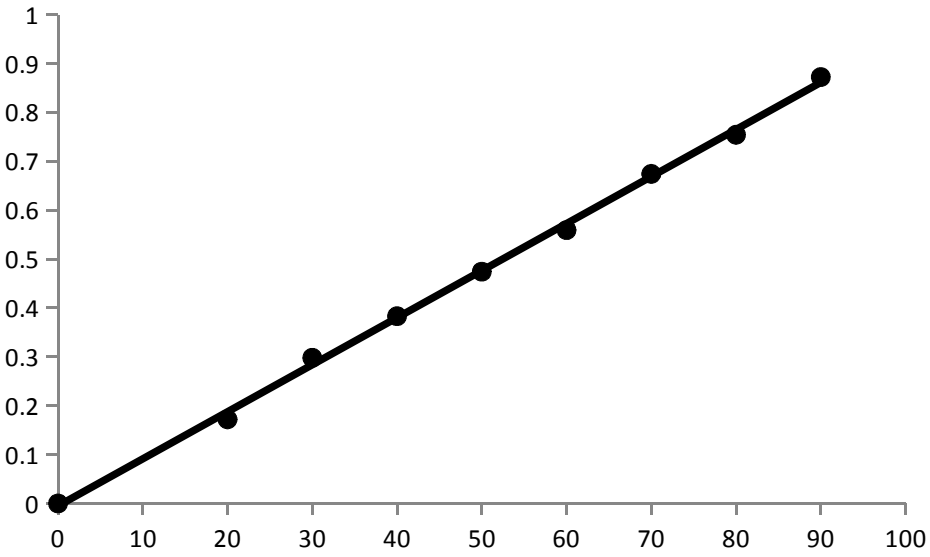


Fig. 16. Calibration graph of ZT with 2, 4-DNP

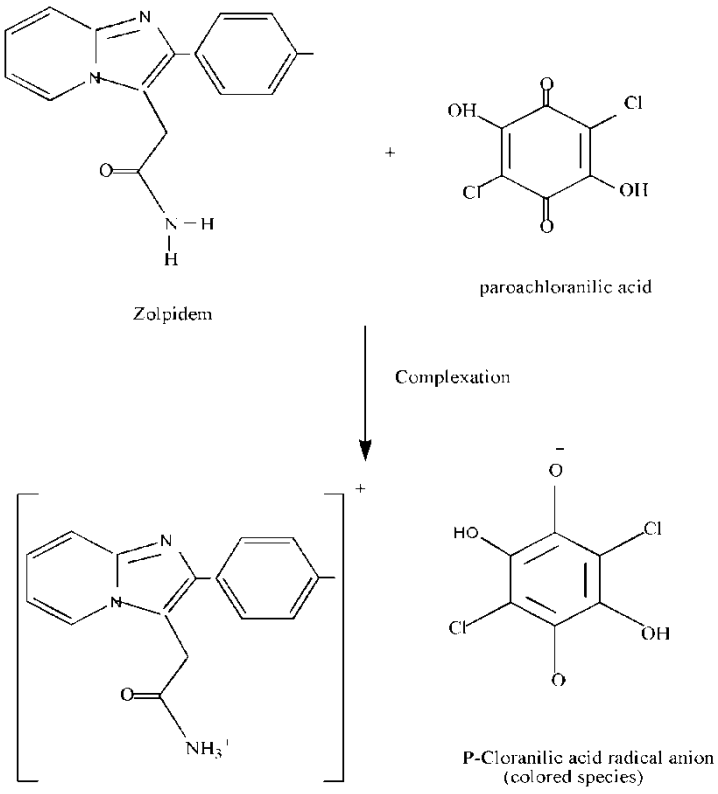
METHOD 9 (ESTIMATION OF ZOLPIDEM BY p-CA)

9.1 PRINCIPLE INVOLVED

In this method the interaction of zolpidem with π-acceptor (p-CA) at room temperature was found to yield colored charge transfer complex **(Scheme 9)** with absorption maximum of 530 nm. In polar solvents, complete electron transfer from zolpidem (D) as an electron donor to the acceptor moiety (A) takes place resulting in the formation of intensely colored radical anions. The reaction sequence can be shown as

$$D + A \rightleftharpoons \underset{\text{Complex}}{(D\text{-}A)} \rightleftharpoons \underset{\text{Radical anions}}{D^{+} + A^{-}}$$

9.2 REACTION INVOLVED



Scheme 9: Reaction of p-CA and ZT

9.3 REAGENTS USED

9.3.1 P-Chloranilic acid (p-CA) 0.2% (w/v)

A 0.2% w/v of the reagent solution was freshly prepared by dissolving 0.2 g of p-CA in 100 ml of methanol.

9.4 PREPARATION OF STANDARD CALIBRATION CURVE

9.4.1 Preparation of standard stock solution

Accurately weighed 100.0 mg of zolpidem (bulk drug) was dissolved in 40.0 ml of methanol in 100 ml volumetric flask and sonicated for about 15min to enhance the solubility and volume was made up to the mark with methanol to obtain final concentration of 1000 µg/ml (Stock solution).

9.4.2 Preparation of calibration curve

Aliquots of zolpidem ranging from 0.2- 2.9 ml (20- 290 µg/ml) were pipette into as series of 10 ml volumetric flask. To each 1 ml of p-CA reagent was added and the volume was made up to mark with methanol. The colored product was formed immediately at room temperature (25±1^{0}C) and absorbance was measured after 2 minutes of mixing at 530 nm against the reagent blank, and the calibration curve and absorption spectra are represented in Fig. 17 and Fig. 18.

9.5 ANALYSIS OF TABLET DOSAGE FORM

10 tablets were weighed and their contents are mixed thoroughly. An accurately weighed portion of powder equivalent to the 100 mg of ZT was weighed into a 100 ml volumetric flask containing about 50 ml of methanol. It was shaken thoroughly for about 5-10 min, filtered thorough whatman filter paper to remove insoluble matter and diluted to the mark with methanol to prepare 1000 µg/ml solution. From the above stock solution 0.5 ml was taken in a 10 ml volumetric flask to obtain a concentration of 50 µg/ml. Then to that solution 1 ml of p-CA reagent was added and the volume was made up to mark with methanol. The colored product was formed immediately at room temperature (25±1^{0}C) and absorbance was measured after 2 minutes.

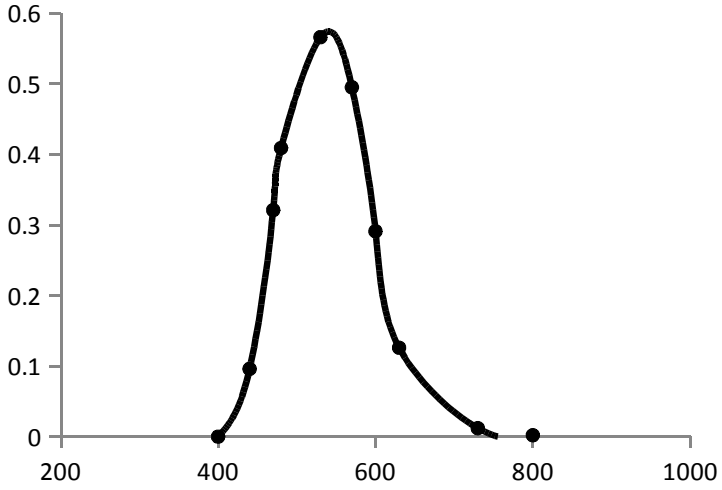


Fig.17. Absorption spectra of p-CA with ZT against the reagent blank

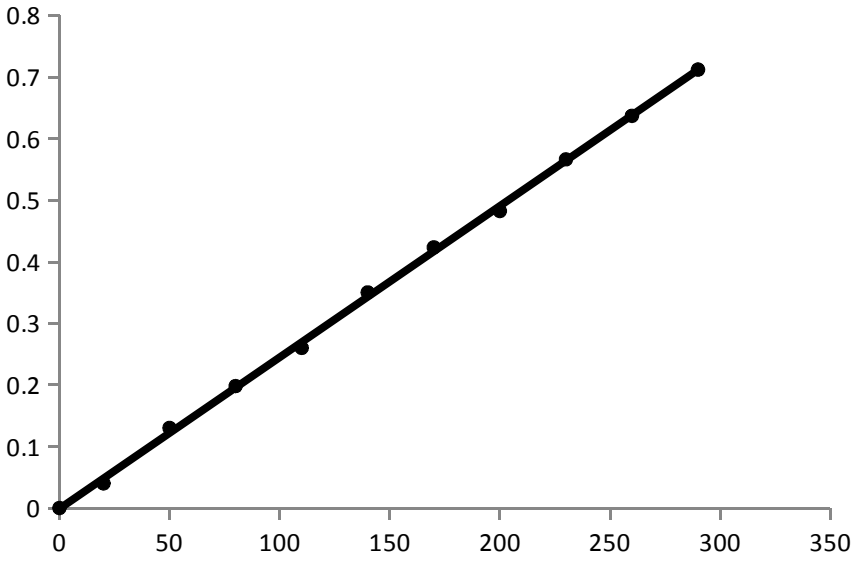


Fig.18. Calibration graph of ZT with p-CA

PART B: GAS CHROMATOGRAPHY METHOD

METHOD 10: (ESTIMATION OF PREGABALIN BY GAS CHROMATOGRAPHY)

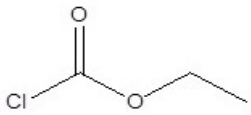
10.1 PRINCIPLE INVOLVED

Pregabalin possesses different functional groups such as carboxylic acid and primary amine group. Ethylchloroformate acts as derivatizing agent. ECF reacts with primary amine group present in pregabalin and forms volatile product and eluted from a capillary GC

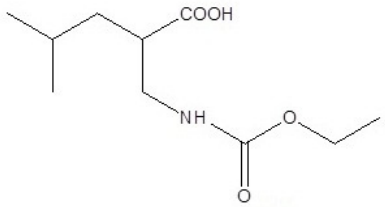
column, each having a single peak. The reaction was carried out in methanol. Aqueous solution of pyridine is used as a reaction medium for better GC response.

10.2 REACTION INVOLVED

(a)



(b)



Scheme 10: Structure of the derivative ECF (a) ECF (b) Dervitized product of PGB with ECF

10.3 INSTRUMENT:

GC studies were carried out on SHIMADZU model 2014 (Shimadzu Technologies, Japan) coupled with a split/split less injector and FID. The computer with GC solutions software has been used to control the gas chromatograph attached with a Cannon laser printer. Rtx-5 capillary column (cross bond 5% diphenyl/ 95% dimethyl polysiloxane) with a length of 30 meters and an internal diameter of 0.25 mm was used throughout the study.

10.4 CHEMICALS AND REAGENTS

Analysis was performed on methanol solutions of PGB. All the standards, ECF, chloroform, pyridine were supplied by Sigma and met Pharamcopoeial requirements. Methanol of analytical quality was procured from MERK (Worli, Mumbai)

10.5 PREPARATION OF STANDARD STOCK SOLUTION

Pregabalin solution at a concentration of 1mg/ml was prepared and diluted to obtain serial dilutions from 2 to10 µg/ml. The solutions were kept below 5^0C and were protected from light.

10.6 CHROMATOGRAPHIC CONDITIONS

The GC-FID parameters used in the method development were based on the boiling point of the drug. The injection port and detector temperature were set to 170˚C and 250˚C, respectively

Manual splitless injection of approximately 2 µl sample was performed at an inlet temperature of 170˚C. The detector temperature was set to 250˚C. After injection, the oven temperature was increased from 80˚C to 160˚C at a rate of 100˚C per min for 5 min. The initial pressure of carrier gas (Nitrogen) was maintained at 83.7 Kpa for 3.5 minutes and pressure was increased at a rate of 20 Kpa/min up to 120 Kpa and held constant for 5 min.

Synthetic air (flow rate of 100 ml/min), hydrogen (25 ml/min) were fed to the FID. All the gases used in these studies were of Pharamcopoeial purity.

PGB analysis was performed after derivatization, PGB is a polar molecule and therefore, a polar solvent methanol was used as the diluent. The capillary column coated with

5% diphenyl/ 95% dimethyl polysiloxane is a good choice for separation of this analyte since they elute as symmetrical peaks at a wide range of concentrations.

10.7 ASSAY

Standard solutions of PGB in HPLC grade methanol, having final concentrations in the range of 2-10 µg/ml were prepared. The drug solution and ECF were added in 1:1 ratio. The solution was heated at 70^0 C for 5 minutes. Solution was evaporated after addition of pyridine and chloroform. Upper layer was collected, redissolved in methanol. 2 µl aliquot of each derivatized sample solution was injected on to the column and the chromatogram (Fig.19.) was recorded. Calibration graph was constructed by plotting the mean peak area as a function of pregabalin concentration (Fig.20.)

10.8 ANALYSIS OF TABLET DOSAGE FORM

Five capsules were weighed and their contents are mixed thoroughly An amount of powder equivalent to the average weight of a capsule was mixed with methanol and shaken for 20 min at a frequency of approximately 3 cycle's/s. The solution was then filtered through 0.2 µm membrane filter paper. The drug solution and ECF were added in 1:1 ratio. The solution was heated at 70^0 C for 5 minutes. Solution was evaporated after addition of pyridine and chloroform. Upper layer was collected, redissolved in methanol. Chromatogram was shown in the (Fig.21.)

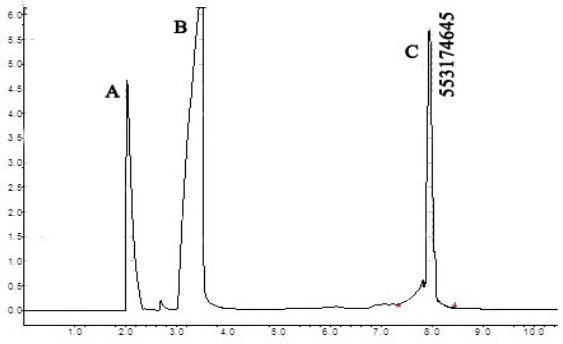


Fig.19. Chromatogram obtained from Pregabalin (PGB) solution, Methanol (A), ethyl chloroformate (B), pregabalin (C)

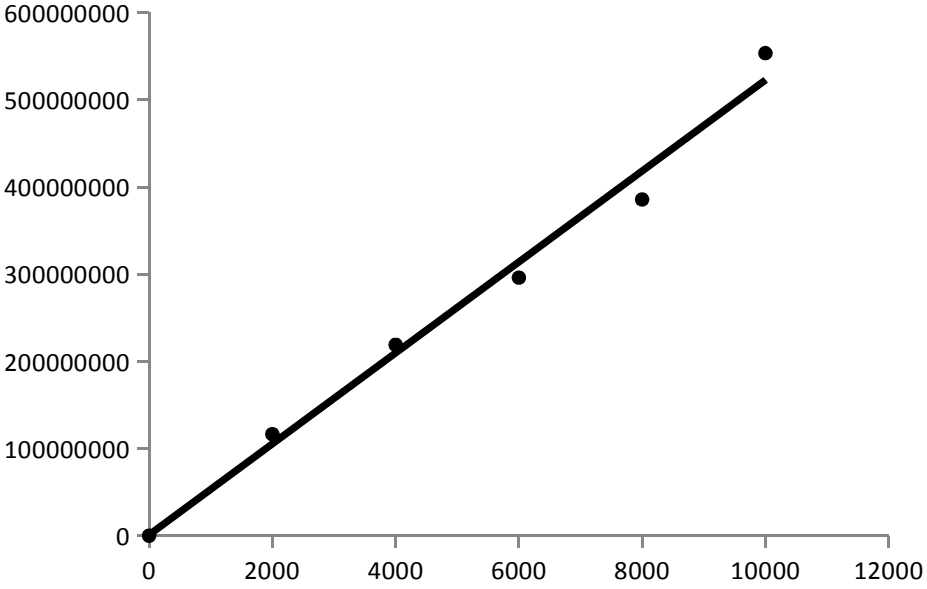


Fig.20. Calibration graph of PGB with ECF

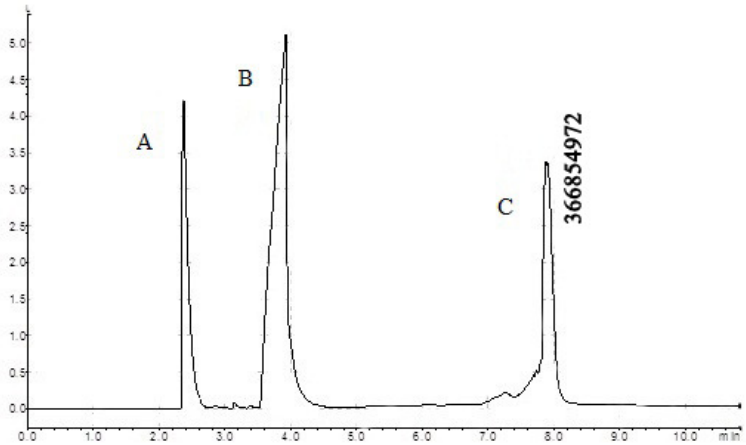


Fig.21. Chromatogram obtained from Pregabalin (PGB) capsule solution. Methanol (A), ethyl chloroformate (B), pregabalin (C)

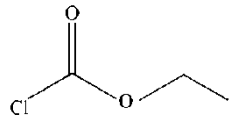
METHOD 11: (ESTIMATION OF ZOLPIDEM BY GAS CHROMATOGRAPHY)

11.1 PRINCIPLE INVOLVED

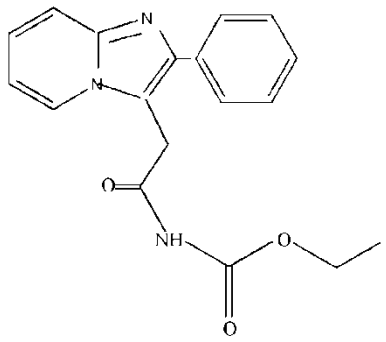
Zolpidem contain acetamide (keto group and amine group) as functional group. Ethylchloroformate acts as derivatizing agent. ECF reacts with secondary amine group present in zolpidem and forms volatile product and elutes from a capillary GC column, each having a single peak. The reaction was carried out in methanol. Aqueous solution of pyridine was used as a reaction medium for better GC response.

11.2 REACTION INVOLVED

(a)



(b)



Scheme 11: Structure of the derivative ECF (a) ECF (b) Dervitized product of zolpidem with ECF.

11.3 PREPARATION OF STANDARD STOCK SOLUTION

Zolpidem solution at a concentration of 1mg/ml was prepared and diluted to obtain serial dilutions from 2 to10 ng/ml. The solutions were kept below 5^0C and were protected from light.

11.4 CHROMATOGRAPHIC CONDITIONS

The GC-FID parameters used in the method development were based on the boiling point of the drug. The injection port and detector temperature were set to 170^0C and 250^0C, respectively.

Manual splitless injection of approximately 2 µl sample was performed at an inlet temperature of 170^0C. The detector temperature was set to 250^0C. After injection, the oven temperature was increased from 80^0C to 180^0C at a rate of 100^0C per min for 5 min. The initial carrier gas pressure (Nitrogen) was maintained at 29.8 Kpa for 3.5 minutes and pressure was increased at a rate of 20 Kpa/min up to 120 Kpa and held constant for 4.50 min.

Synthetic air (flow rate of 100 ml/min), hydrogen (25 ml/min) were fed to the FID. All the gases used in these studies were of Pharamcopoeial purity.

ZT analysis was performed after derivatization, ZT is a polar molecule and therefore, a polar solvent methanol was used as the diluent. The capillary column coated with 5% diphenyl/ 95% dimethyl polysiloxane is a good choice for separation of this analyte since they elute as symmetrical peaks at a wide range of concentrations.

11.5 ASSAY

Standard solutions of ZT in HPLC grade methanol, having final concentrations in the range of 2-10 µg/ml were prepared. The drug solution and ECF were added in 1:1 ratio. The solution was heated at 70^0 C for 5 minutes. Solution was evaporated after addition of pyridine and chloroform. Upper layer was collected, redissolved in methanol. 2 µl aliquot of each derivatized sample solution was injected on to the column and the chromatogram (Fig.22.) was recorded. Calibration graph was constructed by plotting the mean peak area as a function of zolpidem concentration (Fig.23.)

11.6 ANALYSIS OF TABLET DOSAGE FORM

10 tablets were weighed and their contents were mixed thoroughly An amount of powder equivalent to the average weight of a capsule was mixed with methanol and shaken for 20 min at a frequency of approximately 3 cycle's/s. The solution was then filtered through 0.2 µm membrane filter paper. The drug solution and ECF were added in 1:1 ratio. The solution was heated at 70^0 C for 5 minutes. Solution was evaporated after addition of 2 ml pyridine and 2 ml chloroform. Upper layer was collected, redissolved in methanol. Chromatogram is shown in the Fig.24.

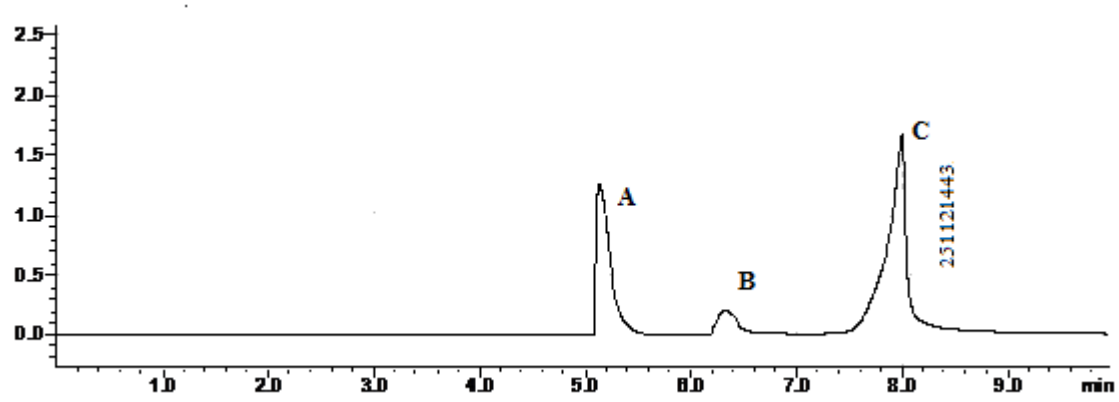


Fig.22. Chromatogram obtained from Zolpidem (ZT) solution, Methanol (A), ethyl chloroformate (B), zolpidem (C)

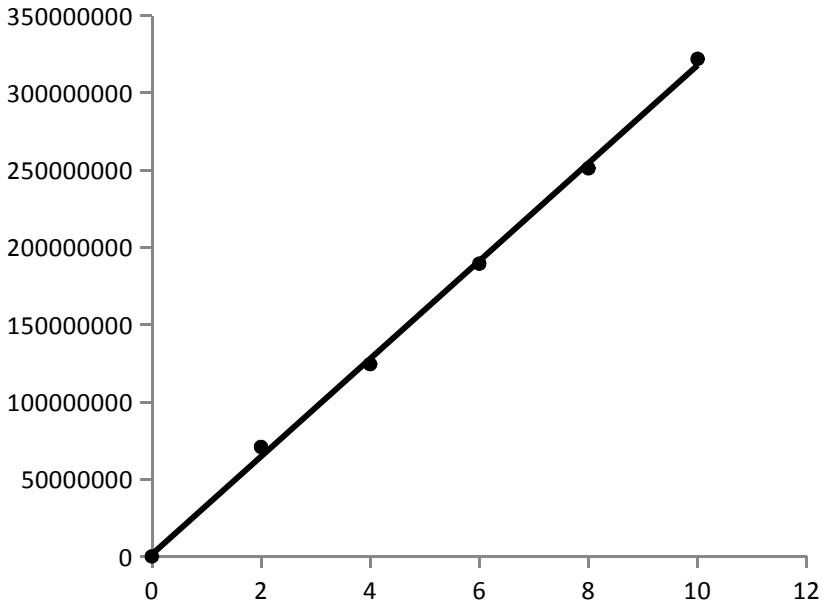
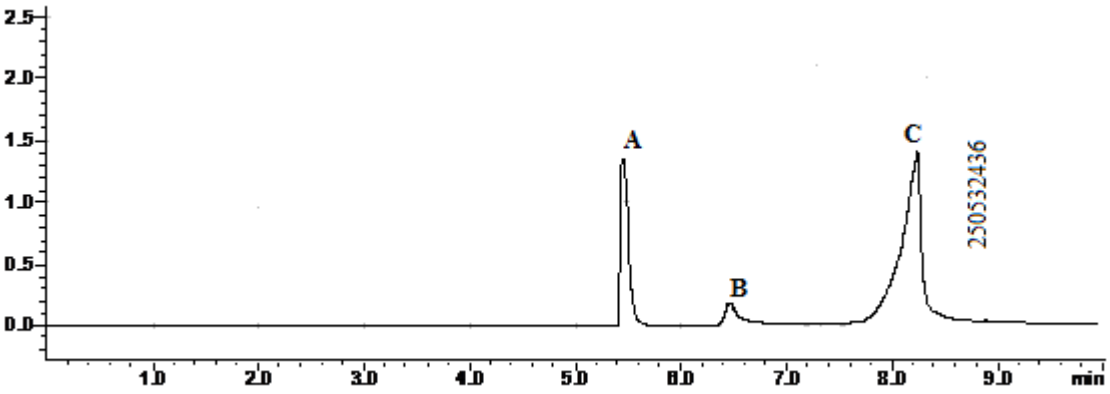


Fig.23. Calibration graph of Zolpidem



RESULTS AND DISCUSSION

PART A: UV/VISIBLE SPECTROMETRIC METODS

Table 1: Optical characters of pregabalin spectrophotometric methods

S. NO	Parameter	NQS	Sangers	MBTH	Gibb's	BMR	2,4-DNP
1.	λ_{max}/ nm	485 nm	353 nm	668 nm	400 nm	471nm	461 nm
2.	Beers law limits (µg/ml)	5-45	5-13	50-350	50-350	50-350	50-450
3.	Molar absorptivity(liter /mol/cm)	$0.17834x10^{-02}$	$0.741x10^{-02}$	4.202×10^{-4}	$3.693x10^{-4}$	$8.862x10^{-4}$	0.273824×10^{-3}
4.	Correlation coefficient (R)	0.993	0.995	0.998	0.999	0.999	0.998
5.	Sandell's sensitivity (g/ml 0.001 abs unit)	0.0546	0.0892	0.378	0.4310	0.862	0.212
6.	Regression equation (Y)	Y=0.018x +0.008	Y=0.057x-0.013	Y=0.0025x +0.0096	Y=0.0021x +0.005	Y=0.0021x+0.05	Y=0.001x-0.007
7.	Slope, *b*	0.018	0.057	0.002	0.002	0.001	0.001
8.	Intercept, a	0.008	0.013	0.0096	0.005	0.003	0.007
9.	µ Relative standard deviation	0.487	0.804	0.35	0.36	0.38	0.25

Table 2: Validation parameters of pregabalin spectrophotometric methods

S.NO	Parameter	NQS	Sangers	MBTH	Gibb's	BMR	2,4-DNP
1	Limit of detection (%g/ml)	0.29	0.24	2.45	2.45	3.99	1.90
2	Limit of quantification(µg/ml)	0.88	0.74	7.44	7.17	12.11	5.77
3	% Intraday RSD	0.238	0.2137	0.32	0.38	0.40	0.25
4	% Interday RSD	0.487	0.804	0.5	0.78	0.73	0.59
5	Standard error of mean	0.0152	0.0035	0.0013	0.0018	0.0004	0.0042

Table 3: Recovery studies of pregabalin spectrophotometric methods

Formulation	Method	Level of % Recovery	Sample PGB µg/ml	Standard PGB µg/ml	Avg amount from calibration graph	Avg %Recovery[a]	% RSD
Pregeb 75	NQS	80%	10	12	21.96	99.53	0.13
		100%	10	15	24.98	99.71	0.16
		120%	10	18	27.97	99.72	0.13
	Sangers	80%	5	4.8	9.78	99.54	0.10
		100%	5	6.0	10.98	99.62	0.14
		120%	5	7.2	12.18	99.59	0.16
	MBTH	80%	50	80	129.5	99.54	0.20
		100%	50	100	149.8	99.66	0.15
		120%	50	120	169.83	99.85	0.22
	Gibbs	80%	50	80	129.6	99.37	0.15
		100%	50	100	149.7	99.43	0.25
		120%	50	120	169.6	99.65	0.16
	BMR	80%	50	80	129.4	99.48	0.23
		100%	50	100	149.6	99.55	0.29
		120%	50	120	169.3	99.36	0.19
	2,4- DNP	80%	50	80	129.8	99.75	0.22
		100%	50	100	149.5	99.30	0.20

		120%	50	120	169.8	99.63	0.15

a = average of three determinations

Table 4: Optical characters of Zolpidem spectrophotometric methods

S.NO	Parameter	MBTH	2,4 DNP	p-CA
1	λ_{max} (nm)	664 nm	416 nm	530 nm
2	Beers law limits (µg/ml)	50-350	20-90	20-290
3	Molar absorptivity (liter /mol/cm)	2.1386×10^{-3}	6.5781×10^{-3}	1.530×10^{-3}
4	Correlation coefficient (R)	0.998	0.998	0.999
5	Sandell's sensitivity (µg/ml 0.001 abs unit)	0.378	0.1162	0.50
6	Regression equation (Y)	Y=0.002x+0.011	Y=0.09x-0.005	Y = 0.002x-0.001
7	Slope, *b*	0.002	0.090	0.002
8	Intercept, a	0.011	0.005	0.001
9	µ Relative standard deviation	0.20	0.41	0.33

Y= bC+a, where C is the concentration of Zolpidem in µg/ml and Y is the absorbance at the respective maximum absorbance

Table 5: Validation parameters of zolpidem spectrophotometric methods

S.NO	Parameters	MBTH	2,4 DNP	p-CA
1	Limit of detection (%g/ml)	3.085	0.836	1.914
2	Limit of quantification(µg/ml)	9.35	2.53	5.80
3	% Intraday RSD	0.50	0.66	0.36
4	% Interday RSD	0.62	0.78	0.54
5	Standard error of mean	0.000477	0.000930	0.000763

Table 6: Recovery studies of Zolpidem spectrophotometric methods

Formulation	Method	Level of % Recovery	Sample ZT µg/ml	Standard ZT µg/ml	Avg amount from calibration graph	Avg %Recovery[a]	% RSD
Zolfresh	MBTH	80%	50	80	129.3	99.37	0.24
		100%	50	100	149.4	99.56	0.21
		120%	50	120	169.2	99.43	0.14
	2,4- DNP	80%	20	24	43.82	99.44	0.18
		100%	20	30	49.80	99.36	0.27
		120%	20	36	55.83	99.48	0.16
	p-CA	80%	20	40	59.80	99.53	0.21
		100%	20	50	69.60	99.46	0.25
		120%	20	60	79.90	99.63	0.15

PART B: GAS CHROTOGRAPHIC METHOD

Table 7: Validation parameters of Pregabalin and Zolpidem Gas chromatographic methods

S.NO	Parameters	Pregabalin	Zolpidem

1	Linearity	2-10 µg/ml	2-10 ng/ml
2	Limit of detection	5.295×10^{-5}	0.0116
3	Limit of quantification	1.605×10^{-4}	0.0350
5	% Intraday RSD	0.0066	0.023
6	% Interday RSD	0.0167	
7	Relative retention time (min)	0.994	

Drug	**Level of % Recovery**	**Sample PGB µg/ml**	**Standard PGB µg**	**Avg amount from calibration graph**	**Avg % Recovery**[a]	**% RSD**
Pregabalin	80%	2	3.2	5.20	100.06	0.042
	100%	2	4	5.99	99.74	0.035
	120%	2	4.8	6.80	100.04	0.045

Table 8: Recovery studies of Pregabalin gas chromatography method

a: average of six replicate determination

Table 9: Recovery studies of Zolpidem gas chromatography method

Drug	Level of % Recovery	Sample ZT ng/ml	Standard ZT ng/ml	Avg amount from calibration graph	Avg % Recovery [a]	% RSD
Zolpidem	80%	2	3.2	5.199	99.90	0.056
	100%	2	4	5.996	99.95	0.042
	120%	2	4.8	6.80	100.13	0.030

a: average of six replicate determination

DISCUSSION

PART A: UV- VISIBLE SPECTROPHOTOMETRY

The optical characteristics such as absorption maxima, Beer's law limits, molar absorptivity, saddle's sensitivity are given in the **Table 1** and **Table 4** for pregabalin and zolpidem respectively. The regression analysis using the least squares was made for the slope (b) intercept (a) and correlation (R) obtained from different concentrations and results are summarized in table 1 and table 4. The percentage relative standard deviation calculated from the six measurements and summarized in **Table 1 and Table 4**. The results showed that the methods have reasonable precision.

Results obtained with the proposed methods confirm the suitability of these methods for pharmaceutical dosage forms. The other active ingredients and excipients usually present in the pharmaceutical dosage forms did not interfere in the estimation when some commercial dosage forms were analyzed by these methods. The accuracy of the method is confirmed by the recovery studies, by adding known amount of the pure drug to the formulation already analyzed by this method and the analytical data presented in **Table 3** and **Table 6** for pregabalin and zolpidem respectively.

In all the above methods, the optimum concentration for the estimation of pregabalin and zolpidem was established by varying one parameter at a time and keeping the other fixed and observing the effect of product on the absorbance of the colored species and incorporated in the procedures. The optimum concentration of pregabalin and zolpidem established by varying drug concentration and by keeping the reagent concentration fixed. After establishing the optimum concentration for drug, the reagent concentration was varied. The above ranges of drug and reagents concentrations were chosen because the colored species formed gave better absorbance and obeyed beer's law satisfactorily.

The methods reported here are found to be simple, sensitive, accurate, precise and economical and can be used in the determination of pregabalin and zolpidem from pharmaceutical dosage forms in a routine manner.

PART B: GAS CHROMATOGRAPHY

The development of analytical method for the determination of drugs by GC has received considerable attention in recent years because of their importance in quality control of drugs and drug products. The objective of this study was to develop a rapid and sensitive GC method for the analysis of pregabalin and zolpidem in bulk and its formulation using the most commonly employed Rtx-5 capillary column with FID detection.

The run time was set at 10.53 min and 10.83 for pregabalin and zolpidem respectively. Pregabalin appeared on the chromatogram at 7.56 min and zolpidem at 8.003 min. When the samples were injected six times, the retention times of the drugs were same. The validated parameters such as linearity, LOD, LOQ, recovery, intra and inter day R.S.D are summarized in **Table 7**. The peak areas of drugs were reproducible as indicated by low coefficient of variance. When the concentration of pregabalin and zolpidem and its respective peak areas were subjected to regression analysis by least squares method, a high correlation coefficient were observed (r^2=0.986 for PGB and r^2=0.998 for ZT) in the range of 2- 10 µg/ml and 2-10 ng/ml for PGB and ZT respectively. The regression equation was used to estimate the amount of pregabalin and zolpidem in formulations or in validation study (precision and accuracy).

The proposed GC methods were also validated for intra and inter day variations. Thus, the results showed that the proposed GC method is highly reproducible. When a known concentration of drug solution was added to preanalyzed samples of commercial dosage forms of pregabalin and zolpidem, there was high recovery of these drugs indicating that the proposed method is highly accurate and the values were presented in **Table 8 and 9**.

The GC method developed in the present study has been used to quantify pregabalin and zolpidem in capsule and tablet dosage form respectively. No interfering peaks were found in the chromatogram indicating that the excipients used in the formulation did not interfere with the estimation of the drug by the proposed GC method.

SUMMARY

Several drugs are available in the form of pharmaceutical formulations to control diseases. Methods of assay for controlling the concentration of these chemicals in the medicine and in the living body are necessary. Pharmaceutical analysis occupies a pivotal role in statutory certification of drugs and their formulations either by the industry or by the regulatory authorities. The complexity of the problem encountered in pharmaceutical analysis coupled with importance of achieving the selectivity, speed, cost, simplicity, precision and accuracy results in new methods of analysis being quickly adopted by pharmaceutical industry.

The ever increasing use of pharmacodynamics and chemotherapeutic agents in pharmaceutical preparations makes their determinations a matter of foremost importance. In

some cases, no precise analytical methods are reported and quite often the reported methods need improvement or changes keeping in view of the advances.

Now a day's many of the new formulations are rushing into the market. In some cases, no precise analytical methods are reported and quite often the reported procedures need improvements or changes keeping in the view of the advances.

Among several instrumental techniques (HPLC, GC, fluorimetry, NMR, mass spectroscopy covering IR, UV and visible regions) available for assay of drugs, visible spectrophotometric methods depend only on the nature of chemical reaction utilized for color development and not on sophistication of the equipment. GC method is highly selective and sensitive compared to spectroscopic or other chromatographic methods. GC method is also cost effective as expensive solvents are not required and it is a versatile tool for qualitative and quantitative analysis of drugs and pharmaceuticals.

Due to the importance of analysis, present analytical method has been developed for some of the widely used drugs such as pregabalin and zolpidem. Hence we planned to develop both GC and spectrophotometric methods.

There is a wide scope for the development of new analytical methods for the assay of the pregabalin and zolpidem. Visible spectrometry (part A) and GC (part B) techniques have been used as a tool in the present thesis work. The above two tools have been used for the development of new analytical methods for the assay of drugs mentioned by exploiting their physical and chemical properties (dependent on basic moieties and functional groups present in each drug).

The contents of the thesis have been divided into eight chapters and appropriate reference have been placed at the end of the eighth chapter (i.e., ninth chapter)

Chapter-1 opens with the introduction giving a brief account of various aspects like chemotherapy, instrumental methods, concepts of spectroscopy, various reagents, types of chromatographic methods, general analytical method development and method validation. The introduction includes brief account on selected drugs.

Chapter- 2 explains the objective of present investigation adopted for selected drug.

Chapter-3 for review of literature gives details on reported methods, clinical studies under different experimental conditions, drug introduction and therapeutic importance of selected drug for present work. Also it gives information about the necessity to investigate new analytical methods for pregabalin and zolpidem for the quantitative estimation.

Chapter-4 contains two parts i.e. part A and part B. Part A gives information regarding visible spectrophotometric method (1 - 9). It briefly explains about principle involved in the method, proposed reaction, reagents and materials used, preparation of standard solutions, preparation of calibration curve, analysis of capsule dosage form. Primary amine group in pregabalin and amide group in zolpidem was exploited in the present investigations for the development of the methods.

In method 1 primary amine group of pregabalin reacts with NQS reagent in alkaline medium at room temperature. The reaction followed the formation of orange colored chromogen exhibiting the absorption maximum at 485 nm and obeys beers law in the concentration range 5-45 µg/ml.

In method 2 primary amine group of pregabalin reacts with Sanger reagent in alkaline medium (buffer pH 9). The reaction results in a yellow coloured complex with absorption maximum at 353 nm. Beer's law is obeyed in the concentration range of 5-13 µg/ml.

In method 3 primary amine group of pregabalin reacts with MBTH reagent in the presence of ferric chloride and forms a green colored product with absorption maximum at 668 nm. Beer's law is obeyed in the concentration range of 50-350 µg/ml.

In method 4 primary amine group of pregabalin reacts with Gibbs reagent. In this method oxidation of the drug with the reagent takes place and forms brown colored product with absorption maximum at 400 nm. Beer's law is obeyed in the concentration range of 50-350 µg/ml.

In method 5 primary amine group of pregabalin reacts with BM reagent. This reaction depends on diazotization of drugs with nitrous acid, to form nitrous acid, to form diazotized compound, followed by its coupling with N-(1-naphthyl) ethylene- diamine dihydrochloride [Bratton-Marshall reagent] to form a violet colored chromogen with absorption maximum of 471 nm. Beer's law is obeyed in the concentration range of 50-350 µg/ml.

In method 6 carboxylic group of pregabalin reacts with 2,4-DNP reagent. In this reaction 2, 4-DNP is oxidized by potassium iodate to give diazonium cation which reacts with drug by electrophilic substitution to give deep colored chromogen with absorption maximum of 461 nm. Beer's law is obeyed in the concentration range of 50-450 µg/ml.

In method 7 amide group of zolpidem reacts with MBTH reagent in the presence of ferric chloride and forms a green colored product with absorption maximum at 664 nm. Beer's law is obeyed in the concentration range of 50-350 µg/ml.

In method 8 keto group of zolpidem reacts with 2,4DNP reagent. In this reaction 2, 4-DNP is oxidized by potassium iodate to give diazonium cation which reacts with drug by electrophilic substitution to give deep colored chromogen with absorption maximum of 461 nm. Beer's law is obeyed in the concentration range of 20-90 µg/ml.

In method 9 amide group of zolpidem reacts with parachloranilic acid (p-CA) at room temperature. p-CA act as a π-acceptor and undergo electron transfer and forms colored charge transfer complex with absorption maximum of 530 nm. Beer's law is obeyed in the concentration range of 20-290 μg/ml.

Part B describes about the gas chromatographic method (10 and 11). It gives information regarding the principle involved in the gas chromatography developed by using derivitizing agent, proposed reaction, instrument, chemicals, reagents, chromatographic conditions, assay and analysis of commercial formulation. Ethylchloroformate is used as a deravitizing agent and reacts with primary amine and secondary amine group present in pregabalin and zolpidem respectively. This reaction makes the compounds more volatile and helps in the elution of peaks with better resolution. Rectilinear relationship was observed between 2-10 μg/ml and 2-10 ng/ml for pregabalin and zolpidem respectively.

Chapter 5 explains the results that are divided into two parts part A and part B. In part A, results obtained in each of the UV-Visible spectrophotometric method for estimation of pregabalin and zolpidem are summarized in **Tables 1-6**. In part B, the results obtained for the gas chromatography determination of pregabalin and zolpidem are summarized in **Table 7-9.**

Chapter 6 consists of discussion which is divided into two parts. Part A discusses about developed visible spectrophotometric methods. Part B discusses about developed GC method.

Chapter 7 explains the conclusion of the proposed methods for the quantitative estimation of pregabalin and zolpidem. A set of eleven methods has been developed for the purpose and these methods are validated in terms of sensitivity, accuracy and precision.

These methods can also be used for the routine determination of pregabalin and zolpidem in bulk drug and pharmaceutical formulations.

Chapter 9 contains the references from which the information of pregabalin and zolpidem and introduction of general methodology was collected.

Chapter 10 contains the list of paper communicated so far to various journals.

CONCLUSION

PREGABALIN

Pregabalin is an anticonvulsant drug used for neuropathic pain and as an adjunct therapy for partial seizures with or without secondary generalization in adults. Chemically pregabalin is [S-[+]-3-isobutyl GABA or (S)-3-(amino methyl)-5-methylhexanoic acid. It binds with high affinity to the alpha2-delta site (an auxiliary subunit of voltage-gated calcium channels) in central nervous system tissues. The drug is available in capsule dosage form.

In previous reports high performance liquid chromatographic methods and only few spectrophotometric methods were developed. Need for the development of simple spectrophotometric methods for its assay is highly demanding. In the view of above fact, some simple analytical methods were planned to develop with sensitivity, accuracy, precision

and economical. Since pregabalin is soluble in distilled water and methanol, a number of spectrophotometric methods can be developed for the quantitative estimation of drug in bulk and pharmaceutical formulations.

In part A set of six simple, sensitive, accurate and precise spectrophotometric methods (methods 1 to 6) has been developed for the purpose. The results expressed in **Table 1-3** for spectrophotometric methods. The striking advantage of all the presently developed methods is that they are economical.

The methods are validated in terms of sensitivity, accuracy and precision.

A. Comparative Sensitivity:

   1 > 2 > 6 > 3 > 4> 5

B. Comparative accuracy:

   3> 2 >1 > 4 > 5 > 6

C. Comparative precision:
   6 > 3 > 4 > 5 > 1 > 2

These methods can be used for the routine determination of pregabalin in bulk drugs and in pharmaceutical formulations.

In part B, in present investigation, we have developed sensitive, precise and accurate gas chromatography method (**method 10**) for the quantitative estimation of pregabalin in its bulk and pharmaceutical formulations. The results expressed in **Table 7 and 8** for gas chromatography is promising. The advantage of the developed GC method is that it is sensitive and precise. As only small quantity of solvents is used the method is proved to be economical.

This method can be used for the routine determination of pregabalin in bulk drugs and pharmaceutical formulations.

ZOLPIDEM

Zolpidem is a prescription medication used for the short-term treatment of insomnia, as well as some brain disorders. Chemically zolpidem is N, N, 6-trimethyl-2-p-tolyl-imidazole (1,2-a) pyridine-3-acetamide L-(+)-tartrate. It is a short-acting nonbenzodiazepine hypnotic that potentiates gammaaminobutyric acid (GABA), an inhibitory neurotransmitter, by binding to $GABA_A$ receptors at the same location as benzodiazepines. The drug is available in tablet dosage form.

It is official in British Pharmacopoeia. A HPLC method has been reported in the British pharmacopoeia. Need for the development of simple spectrophotometric methods for its assay is highly demanding.

Here three simple sensitive precise and accurate UV-visible spectrophotometric methods have been established for the quantitative estimation of zolpidem in its bulk and pharmaceutical formulations. The results are given in **Table 4-6.** The methods are validated in terms of sensitivity, accuracy and precision.

A. Comparative Sensitivity
   2 > 1 > 3
B. Comparative accuracy:
   3 >1 > 2
C. Comparative precision:

   1 > 3 > 2

These methods can be used for the routine determination of pregabalin in bulk drugs and in pharmaceutical formulations.

In part B, in present investigation, we have developed sensitive, precise and accurate Gas chromatography method (**method 11**) has been established for the quantitative estimation of pregabalin in its bulk and pharmaceutical formulations. The results expressed in **Table 7** and **9** for gas chromatography is promising. The advantage of this developed GC method is, it is sensitive.

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