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DEVELOPMENT AND VALIDATION OF NEW ANALYTICAL METHOD FOR THE ESTIMATION OF FLUPENTIXOL DIHYDROCHLORIDE IN PURE AND PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

Flupentixol dihydrochloride

In the present work, five simple, sensitive and specific methods (Zero, First, Second order derivative Spectroscopy, RP-HPLC and HPTLC) have been developed for the quantitative estimation of Flupentixol dihydrochloride in bulk and pharmaceutical formulations.

PART A: UV SPECTROPHOTOMETRY

METHOD A: ZERO ORDER DERIVATIVE SPECTROSCOPY

A simple, accurate and precise Zero order derivative Spectroscopy method was developed and validated for the estimation of Flupentixol dihydrochloride in pharmaceutical dosage forms. The stock solution was prepared by weighing 100 mg of Standard Flupentixol dihydrochloride in 100 ml volumetric flask with distilled water. The final stock solution was made to produce 100 mcg / ml with distilled water. Further dilutions were prepared as per procedure. The drug solution showed the maximum absorbance at 229 nm. The linearity was found in the concentration range of 3-15 mcg / ml. The Correlation coefficient was 0.9999. The regression equation was found to be Y = 0.0612 C + 0.0046. The method was validated for linearity, accuracy, precision, limit of detection, limit of quantitation and ruggedness. The limit of detection and limit of quantitation for estimation of Flupentixol dihydrochloride was found to be 0.128 (mcg / ml) and 0.390 (mcg / ml), respectively. Recovery of Flupentixol dihydrochloride was

found to be in the range of 98.63 – 99.87 %.

METHOD B: FIRST ORDER DERIVATIVE SPECTROSCOPY

A simple, specific, accurate and precise First order derivative Spectroscopy method was developed and validated for the estimation of Flupentixol dihydrochloride in pharmaceutical dosage forms. The stock solution was prepared by weighing 100 mg of Standard Flupentixol dihydrochloride in 100 ml volumetric flask with distilled water. The final stock solution was made to produce 100 mcg / ml with distilled water. Further dilutions were prepared as per procedure. The drug solution showed the maximum absorbance at 222 nm. The linearity was found in the concentration range of 3-15 mcg / ml. The Correlation coefficient was 0.9996. The regression equation was found to be Y = 0.0158 C - 0.0015. The method was validated for linearity, accuracy, precision, limit of detection, limit of quantitation and ruggedness. The limit of detection and limit of quantitation for estimation of Flupentixol dihydrochloride was found to be 0.12 (mcg / ml) and 0.37 (mcg / ml), respectively. The % RSD values were less than 2. Recovery of Flupentixol dihydrochloride was found to be in the range of 99.21 - 100.87 %.

METHOD C: SECOND ORDER DERIVATIVE SPECTROSCOPY

A simple, specific, accurate and precise Second order derivative Spectroscopy method was developed and validated for the estimation of Flupentixol dihydrochloride in pharmaceutical dosage forms. The stock solution was prepared by weighing 100 mg of Standard Flupentixol dihydrochloride in 100 ml volumetric flask with methanol. The final stock solution was made to produce 100 mcg / ml with distilled water.

Further dilutions were prepared as per procedure. The drug solution showed the maximum absorbance at 214 nm. The linearity was found in the concentration range of 3-15 mcg / ml. The Correlation coefficient was 0.9998. The regression equation was found to be Y = 0.0036 C + 0.0001. The method was validated for linearity, accuracy, precision, limit of detection, limit of quantitation and ruggedness. The limit of detection and limit of quantitation for estimation of Flupentixol dihydrochloride was found to be 0.36 (mcg / ml) and 1.11 (mcg / ml), respectively. The % RSD values were less than 2. Recovery of Flupentixol dihydrochloride was found to be in the range of 99.18 – 99.87 %.

PART B: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

A simple, specific, accurate, and precise reverse phase high performance liquid

chromatographic (RP-HPLC) method was developed and validated for the estimation of Flupentixol dihydrochloride in pharmaceutical dosage forms. A Phenomenex - Gemini C -18 (250 x 4.6 mm, 5 µm) in isocratic mode, with mobile phase containingMethanol: Water (65: 35, v / v) was used. The flow rate was 1 ml / min and effluentswere monitored at 229 nm. Chromatogram showed the main peak at a retention timeof 4.28 min. The method was validated for linearity, accuracy, precision, limit of detection, limit of quantitation, robustness and ruggedness. The linearity was found tobe in the range of 10 to 50 mcg / ml. The limit of detection and limit of quantitation for estimation of Flupentixol dihydrochloride was found to be 0.11 (mcg / ml) and 0.34 (mcg / ml), respectively. Recovery of Flupentixol dihydrochloride was found tobe in the range of 98.95 - 99.88 %.

PART C: HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

A simple, specific, accurate and precise high performance thin layer chromatographymethod has been developed for determination of Flupentixol dihydrochloride in bulk and pharmaceutical dosage forms. The method uses aluminium plates coated with silica gel 60 F_{254} as stationary phase and toluene: glacial Acetic Acid (7: 3, v / v) as mobile phase. Densitometric evaluation of the separated bands was performed at 229 nm. The R_F value of Flupentixol dihydrochloride was 0.21 ± 0.02. The validatedcalibration range was 300-1500 ng per spot (r2 = 0.9993). The limit of detection was 6.24 ng / spot and Limit of quantitation was 18.92 ng / spot. Results of analysis were validated statistically and by recovery studies. The method was validated according tothe ICH guidelines with respect to linearity, accuracy, precision and ruggedness.

KEYWORDS: Flupentixol dihydrochloride, Method validation, Zero order derivative Spectroscopy,First order derivative Spectroscopy, Second order derivative Spectroscopy, RP-HPLC, HPTLC and ICH guidelines.

CHAPTER 1

INTRODUCTION

Pharmaceutical Analysis[1,2] is the branch of chemistry involved in separating, identifying and determining the relative amounts of the components making up a sample of matter. It is mainly involved in the qualitative identification or detection of compounds and quantitative measurements of the substances present in bulk and pharmaceutical preparation.

Pharmaceutical analysis derives its principles from various branches of science like Chemistry, Physics, Microbiology, Nuclear Science, Electronics, etc. Analytical method is a specific application of a technique to solve an analytical problem.

Analytical instrumentation plays an important role in the production and evaluation of new products and in the protection of consumers and the environment. This instrumentation provides the lower detection limits required to assure safe foods, drugs, water and air.

TYPES

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.

(b) 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, the characteristic movement of a substance through a defined medium under controlled conditions, (c) electrical measurement, (d) measurement of some spectroscopic properties of the compound.

Analytical method development[3]

Pharmaceutical products formulated with more than one drug, typically referred to as combination products, are intended to meet previously unmet patients need by combining the therapeutic effects of two or more drugs in one product. These combination products can present daunting challenges to the analytical chemist responsible for the development and validation of analytical methods. This presentation will discuss the development and validation of analytical method (Spectrophotometric, High performance liquid chromatography (HPLC), & High performance thin layer chromatography (HPTLC)) for drug products containing more than one active ingredient. The official test methods that

result from these processes are used by quality control laboratories to ensure the identity, purity, potency, and performance of drug products.

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

Basic criteria for new method development of drug analysis.

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

UV – VISIBLE SPECTROPHOTOMETRY[4,5,6]

Spectrophotometry, one of the valuable techniques in pharmaceutical analysis is defined as the method of analysis, which deals with the measurement of spectra. Spectrophotometry is a branch, which embraces the measurement of absorption of radiation energy of definite and narrow wavelength approximating monochromatic radiations by chemical species. Absorption Spectrophotometry is the measurement of the absorption of electromagnetic radiation of definite and narrow wavelength range by molecules, ions and atoms of a chemical substance. Technique most commonly employed in analytical field includes

ultraviolet, visible, infrared and atomic absorption spectroscopy.

This deals with the absorption of electromagnetic radiation in the wavelength region of160 to 780 nm. UV absorption spectroscopy deals with absorption of light by a sample in the Ultra Violet (UV) region (190-380 nm), while Visible region (380-780 nm) absorption spectroscopy (colorimetry) deals with absorption of light by a sample in the visible region (380-780 nm). Absorption of UV – Visible light causes promotionof a valence electron from bonding to antibonding orbitals.

Derivative Spectrophotometry[5-7]

Derivative Spectrophotometry involves the conversion of a normal spectrum to its first, second or higher derivative spectrum and are shown in **Fig: 1.1.** In the context of Derivative Spectrophotometry, the normal absorption is referred to as fundamental, Zero order or D^0 spectrum.

The First derivative spectrum (D^1) is a plot of the rate of change of absorbance with wavelength against wavelength, i.e. a plot of the slope of the fundamental spectrum against wavelength or a plot of $dA / d\lambda$ versus λ. The λ_{max} is a wavelength of zero slope and gives $dA / d\lambda = 0$, i.e. a cross-over point, in the D^1 spectrum.

The Second derivative spectrum (D^2) is a plot of the curvature of the D^0 spectrum against wavelength or a plot of $d^2A / d\lambda^2$ versus λ. The maximum negative curvature at λ_{max} in the D^0 spectrum gives a minimum in the D^2 spectrum.

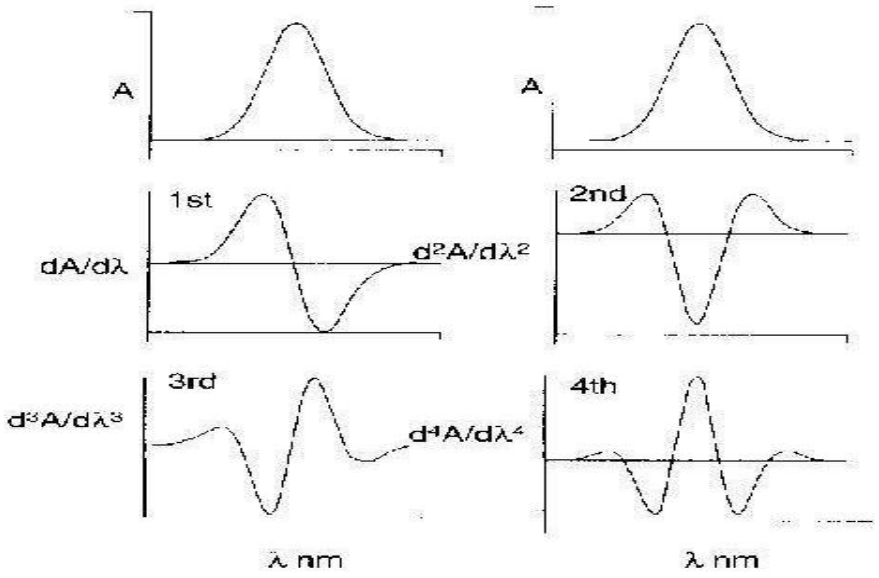


Fig: 1.1: Zero, First, Second, Third and Fourth order derivative curves[8]

In summary the First derivative spectrum of an absorption band is characterized by a maximum, a minimum, and a cross-over point at the λ_{max} of the absorption band. The Second derivative spectrum of an absorption band is characterized by two satellite maxima and an inverted band of which the minimum corresponds to the λ_{max} of the fundamental band.

The Second and Fourth order derivatives have a central peak which is sharper than the original band but of the same height; its sign alternates with increasing order. It is clear that resolution is improved in the even-order spectra, and this offers the possibility of separating two absorption bands which may in fact merge in the Zero - order spectrum. Thus, a mixture of two substances gave a Zero-order spectrum showing no well-defined absorption bands, but the second-order spectrum deduced from this curve showed well resolved peaks.

The Second-order plot of the mixture is identical with that of pure substance. When the interference spectrum can be described by an n^{th} -order polynomial, the interference is eliminated in the (n + 1) derivative.

For quantitative measurements peak heights (expressed in mm) are usually measuredof the long-wave peak satellite of either the second- or fourth-order derivative curves, or for the short-wave peak satellite of the same curves. Derivative spectra can be recorded by means of a wavelength modulation device in which beams of radiation differing in wavelength by a small amount (1-2 nm) fall alternately on the sample cell and the difference between the two readings is recorded. In an alternative procedure, a derivative unit involving resistance/capacitance circuits, filters and operational amplifier are attached to the Spectrophotometer, but as already indicated, derivative curves are most readily obtained by computer-based calculations.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY[9]

In 1903, Mikhail Tswett discovered Chromatography technique. It serves as a means of resolution of mixtures. The name suggests ***chroma*** meaning “colour” and ***graphein*** meaning “write”.

HPLC is one of the types of Chromatography. In modern pharmaceutical industries, HPLC is the major and integral analytical tool applied in all stages o f drug discovery, development,

and production. Effective and fast method development is of paramount importance throughout this drug development life cycle. This requires a thorough understanding of HPLC principles and theory which lay a solid foundation for appreciating the many variables that are optimized during fast and effective HPLC method development and optimization.

Principle[10]

The principle of separation is normal phase mode and reverse phase mode is adsorption. When mixtures of components are introduced in to a HPLC column, they travel according to their relative affinities towards the stationary phase. The component which has more affinity towards the adsorbent travels slower.

The component which has less affinity towards the stationary phase travels faster. Since no two components have the same affinity towards the stationary phase, the components are separated.

Most of the drugs in multicomponent dosage forms can be analyzed by HPLC method because of the several advantages like rapidity, specificity, accuracy, precision and ease of automation in this method. HPLC method eliminates tedious extraction and isolation procedures.

Modes of separation in HPLC[9,11]

They are

- Normal phase mode,
- Reversed phase mode,
- Ion exchange chromatography,
- Ion pair chromatography,
- Affinity chromatography and
- Size exclusion chromatography.

In the ***normal phase mode***, the stationary phase is polar and the mobile phase is nonpolar in nature. In this technique, nonpolar compounds travel faster and are eluted first. This is because of the lower affinity between the nonpolar compounds and the stationary phase. Polar compounds are retained for longer times because of their higher affinity with the stationary phase. These compounds, therefore take more times to elute. Normal phase mode of separation is therefore, not generally used for pharmaceutical applications because most

of the drug molecules are polar in natureand hence take longer time to elute.

Reversed phase mode is the most popular mode for analytical and preparative separations of compound of interest in chemical, biological, pharmaceutical, food and biomedical sciences. In this mode, the stationary phase is nonpolar hydrophobic packing with octyl or octa decyl functional group bonded to silica gel and the mobile phase is polar solvent. An aqueous mobile phase allows the use of secondary solute chemical equilibrium (such as ionization control, ion suppression, ion pairing and complexation) to control retention and selectivity. The polar compound gets eluted first in this mode and nonpolar compounds are retained for longer time. As most of the drugs and pharmaceuticals are polar in nature, they are not retained for longer times and hence elute faster. The different columns used are octa decyl silane (ODS) or C_{18}, C_8, C_4, etc., (in the order of increasing polarity of the stationary phase).

In *Ion exchange chromatography*, the stationary phase contains ionic groups like $^+$ $^-$ $_3$ or SO_3, which interact with the ionic groups of the sample molecules. This is suitable for the separation of charged molecules only. Changing the pH and saltconcentration can modulate the retention.

Ion pair chromatography may be used for the separation of ionic compounds and this method can also substitute for ion exchange chromatography. Strong acidic and basic compounds may be separated by reversed phase mode by forming ion pairs (coulumbic association species formed between two ions of opposite electric charge) with suitable counter ions. This technique is referred to as reversed phase ion pair chromatography or soap chromatography.

Affinity chromatography uses highly specific biochemical interactions for separation. The stationary phase contains specific groups of molecules which can adsorb the sample if certain steric and charge related conditions are satisfied. This technique can be used to isolate proteins, enzymes as well as antibodies from complex mixtures.

Size exclusion chromatography separates molecules according to their molecular mass. Largest molecules are eluted first and the smallest molecules last. This methodis generally used when a mixture contains compounds with a molecular mass difference of at least 10%. This mode can be further subdivided into gel permeation chromatography (with organic solvents) and gel filtration chromatography (with aqueous solvents).

Minimum requirement for HPLC[12]

Temperature

Room temperature is the first choice. Elevated temperatures are sometimes used to reduce column pressure are enhancing selectivity. Typically, temperatures in excess of 60^{o}C are not used.

Retention time mechanism

In general, HPLC is a dynamic adsorption process. Analyte molecules, while moving Through the porous packing bead, tend to interact with the surface adsorption sites. Depending on the HPLC mode, the different types of the adsorption forces may be Included in the retention process.

- Hydrophobic (non-specific) interactions are the main ones in Reversed- Phase Separations.
- Dipole-dipole (polar) interactions are dominated in normal phase mode.
- Ionic interactions are responsible for the retention in ion-exchange Chromatography. All these interactions are competitive. Analyte molecules are competing with the eluent molecules for the adsorption sites. So, the stronger analyte molecules interact with the surface and the weaker the eluent interaction, the longer analyte will be retained on the surface.

System Suitability Tests For Chromatographic Methods[13]

Definition

The purpose of the system suitability test is to ensure that the complete testing system (including instrument, reagents, columns, analysts) is suitable for the intended application. The USP Chromatography General Chapter states.

"System suitability tests are an integral part of gas and liquid chromatographic methods. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analysed constitute an integral system that can be evaluated as such."

System suitability is the checking of a system to ensure system performance before or during the analysis of unknowns. Parameters such as plate count, tailing factors, resolution and

reproducibility (% RSD of retention time and area for six repetitions) are determined and compared against the specifications set for the method. These parameters are measured during the analysis of a system suitability "sample" that is a mixture of main components and expected by-products.

System Suitability Parameters and Recommendations.

Parameter	Recommendation
Capacity Factor (k')	The peak should be well-resolved from other peaks and the void volume, generally k' > 2.0
Repeatability	RSD < / = 1 % for N > / = 5 is desirable.
Relative retention	Not essential as long as the resolution is stated.
Resolution (R_S)	R_S of > 2 between the peak of interest and the closesteluting potential interferent (impurity, excipient, degradation product, internal standard, etc.)
Tailing Factor (T)	T of < / = 2
Theoretical Plates (N)	In general should be > 2000

If the results are adversely affected by the changes in column performance (e.g. unacceptable precision of results due to overlapping peaks), the system suitability results from these experiments will help to determine the limits for system suitability criteria. Lists of the terms to be measured and their recommended limits obtained from the analysis of the system suitability sample are given below.

H PERFORMANCE THIN LAYER CHROMATOGRAPHY[14]

Thin layer chromatography (TLC); also known as planar-chromatography or flatbed chromatography is like all other chromatographic techniques, a multi-stage distribution process. HPTLC is the most simple separation technique today availableto the analyst. It can be considered a time machine that can speed your work and allows you to do many things at a time usually not possible with other analytical techniques.

PARAMETERS	HPTLC	TLC
Layer of Sorbent	100µm	250 µ m
Efficiency	High due to smaller particle size generated	Less
Separations	3 - 5 cm	10 – 15 cm
Analysis Time	Shorter migration distance and the analysistime is greatly reduced	Slower
Solid support	Wide choice of stationary phases like silicagel for normal phase and C-8 , C-18 for reversed phase modes	Silica gel ,Alumina &Kiesulguhr
Development chamber	New type that require less amount of mobilephase	More amount
Sample spotting	Auto sampler	Manualspotting
Scanning	Use of UV / Visible / Fluorescence scanner scans the entire chromatogram qualitativelyand quantitatively and the scanner is an advanced type of densitometer	Not possible

TLC / HPTLC are often found more troublesome than GLC / HPLC as quantitative TLC is an off-line technique, hence automation is difficult and because of its open character, is highly influenced by environmental factors. It is, therefore, essential that each step which may require specific approach must be carefully validated to determine potential source of error.

Steps involved in HPTLC[15]

1. Selection of chromatographic layer
2. Sample and standard preparation
3. Layer pre-washing
4. Layer pre-conditioning
5. Application of sample and standard
6. Chromatographic development
7. Detection of spots
8. Scanning
9. Documentation of chromatic plate

Parameters that are affected by the changes in chromatographic conditions are.

1. Retention factor (R_F),
2. Peak purity.

1. Retention factor (R_F)**:** Retention factor (R_F) is defined as the amount of separation due to the solvent migration through the sorbent layer as shown in the formula. It depends on time of development and velocity coefficient or solvent front velocity.

Migration distance of substance

R_F = Migration distance of solvent front from origin

2. Peak purity: The null hypothesis "these spectra are identical" can in this case (purity) with two sided significance. During the purity test the spectrum taken at the first peak slope is correlated with the spectrum of peak maximum [r (s, m)] and the correlation of the spectra taken at the peak maximum with the one from the downslope or peak end [r (m, e)] which is used as a reference spectra for statistical calculation. An error probability of 1 % only is rejected if the test value is greater than or equal to 2.576.

Validation of Analytical Method

"Validation is the process of providing documented evidence that the method does what it is intended to do." In other words, the process of method validation ensures that the proposed analytical methodology is accurate, specific, reproducible, and rugged for its intended use.[16]

Validation is an act of proving that any procedure, process, equipment, material, activity or system performs as expected under given set of conditions and also give the required accuracy, precision, sensitivity, ruggedness, etc.

When extended to an analytical procedure, depending upon the application, it means that a method works reproducibly, when carried out by same or different persons, in same or different laboratories, using different reagents, different equipments, etc.

Advantages of Analytical Method Validation:

The biggest advantage of method validation is that it builds a degree of confidence, not only for the developer but also to the user. Although the validation exercise may appear costly and time consuming, it results inexpensive, eliminates frustrating Repetitions and leadsto better time management in the end.

Minor changes in the conditions such as reagent supplier or grade, analytical setup are unavoidable due to obvious reasons but the method validation absorbs the shock of such conditions and pays for more than invested on the process.

Validation Parameters[17,18,19]

These parameters are termed "analytical performance characteristics" or sometimes "analytical figures of merit."

The various validation parameters are

1) Accuracy,
2) Precision (Repeatability and Reproducibility),
3) Linearity and Range,
4) Limit of detection (LOD) / Limit of quantitation (LOQ),
5) Selectivity / Specificity,
6) Robustness / Ruggedness,
7) Stability and System suitability studies.

(1) Accuracy

The accuracy of an analytical method may be defined as a closeness of the test results obtained by the method to the true value. It is the measure of the exactness of the analytical method developed. Accuracy may often express as percent recovery by the assay of a known amount of analyte added.

Accuracy may be determined by applying the method to samples or mixtures of excipients to which known amount of analyte have been added both above and below the normal levels expected in the samples. Accuracy is then calculated from the test results as the percentage of the analyte recovered by the assay. Dosage form assays commonly provide accuracy within 3-5% of the true value. The ICH documents recommend that accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels, covering the specified range (i.e. three concentrations and three replicated of each concentration).

(2) Precision

The precision of an analytical method is the degree of agreement among individual test results, when the method is applied repeatedly to multiple samplings of homogenous samples. This is usually expressed as the standard deviation or the relative standard deviation (coefficient of variation). Precision is a measure of the degree of reproducibility or of the repeatability of the analytical method under normal operating circumstances.

Repeatability involves analysis of replicates by the analyst using the same equipment and method and conducting the precision study over short period of time while reproducibility involves precision study at Different Occasions, Different Laboratories, Different Batch of Reagent, Different Analysts and Different Equipments.

Determination of Repeatability: - Repeatability can be defined as "the precision of the procedure when repeated by same analyst under the same operating conditions (same reagents, equipments, settings and laboratory) over a short interval of time".

It is normally expected that at least six replicates be carried out and a table showing each individual result provided from which the mean, standard deviation and co -efficient of variation should be calculated for set of n value. The RSD values are important for showing degree of variation expected when the analytical procedure is repeated several time in a standard situation. (RSD below 1% for built drugs, RSD Below 2% for assays in

finished product).

The ICH documents recommend that repeatability should be assessed using a minimum of nine determinations covering the specified range for the procedure (i.e. three concentrations and three replicates of each concentration or using a minimum of six determinations at 100 % of the test concentration).

Determination of reproducibility*:* **-** Reproducibility means the precision of the procedure when it is carried out under different conditions usually in different laboratories on separate, putatively identical samples taken from the same homogenous batch of material. Comparisons of results obtained by different analysts, by the use of different equipments, or by carrying out the analysis at different timescan also provide valuable information.

(3) Linearity and range

The linearity of an analytical method is its ability to elicit test results that are directly (or by a well-defined mathematical transformation) proportional to the analyte concentration in samples within a given range. Linearity usually expressed in terms of the variance around the slope of regression line calculated according to an established mathematical relationship from test results obtained by the analysis of samples with varying concentrations of analyte.

The linear range of detectability that obeyed Beer's law is dependent on the compound analysed and the detector used. The working sample concentration and samples tested for accuracy should be in the linear range. The claim that the method is linear is to be justified with additional mention of zero intercept by processing data by linear least square regression. Data is processed by linear least square regression declaring the regression co-efficient and b of the linear equation y = ax + b together with the correlation coefficient of determination. For the method to be linear the value should be close to 1.

The range of an analytical method is the interval between the upper and lower levelsof the analyte (including these levels) that have been demonstrated to be determined with precision, accuracy and linearity using the method as written.

(4) Limit of detection and Limit of quantitation

Limit of detection: **-** The limit of detection is the parameter of limit tests. It is the lowest level of analyte that can be detected, but not necessarily determined in a quantitative fashion, using

a specific method under the required experimental conditions. The limit test thus merely substantiates that the analyte concentration is above or below a certain level.

The determination of the limit of detection of instrumental procedures is carried out by determining the signal-to-noise ratio by comparing test results from the samples with known concentration of analyte with those of blank samples and establishing the minimum level at which the analyte can be reliably detected. A signal-to-noise ratio of 2:1 or 3:1 is generally accepted.

The signal-to-noise ratio is determined by dividing the base peak by the standard deviation of all data points below a set threshold. Limit of detection is calculated by taking the concentration of the peak of interest divided by three times the signal-to- noise ratio.

For spectroscopic techniques or other methods that rely upon a calibration curve for quantitative measurements, the IUPAC approach employs the standard deviation of the intercept (S_a) which may be related to LOD and the slope of the calibration curve, b, by.

$$LOD = \frac{3.3\ Sa}{b}$$

Limit of quantitation: - Limit of quantitation is a parameter of quantitative assays for low levels of compounds in sample matrices such as impurities in bulk drugs and degradation products in finished pharmaceuticals. The limit of quantitation is thelowest concentration of analyte in a sample that may be determined with acceptable accuracy and precision when the required procedure is applied.

It is measured by analysing samples containing known quantities of the analyte and determining the lowest level at which acceptable degrees of accuracy and precision are attainable. Where the final assessment is based on an instrumental reading, the magnitude of background response by analyzing a number of blank samples and calculating the standard deviation of this response. The standard deviation multiplied by a factor (usually 10) provides an estimate of the limit of quantitation. In many cases, the limit of quantitation is approximately thrice the limit of detection.

$$LOQ \quad \frac{10\ Sa}{b}$$

(5) Selectivity and Specificity

The selectivity of an analytical method is its ability to measure accurately and specifically the analyte of interest in the presence of components that may be expected to be present in the sample matrix.

If an analytical procedure is able to separate and resolve the various components of a mixture and detect the analyte qualitatively the method is called selective. On the other hand, if the method determines or measures quantitatively the component of interest in the sample matrix without separation, it is said to be specific. Hence one basic difference in the selectivity and specificity is that, while the former is restricted to qualitative detection of the components of a sample, the latter means quantitative measurement of one or more analyte.

(6) Robustness and Ruggedness

Robustness: - The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variation in method parameters and provides an indication of its reliability during normal usage. The determination of robustness requires that methods characteristic are assessed when one or more operating parameter varied.

Ruggedness:- 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 normal test conditions such as different laboratories, different analysts, using operational and environmental conditions that may differ but are still within the specified parameters of the assay. The testing of ruggedness is normally suggested when the method is to be used in more than one laboratory. Ruggedness is normally expressed as the lack of the influence on the test results of operational and environmental variables of the analytical method.

(7) Stability and System suitability tests

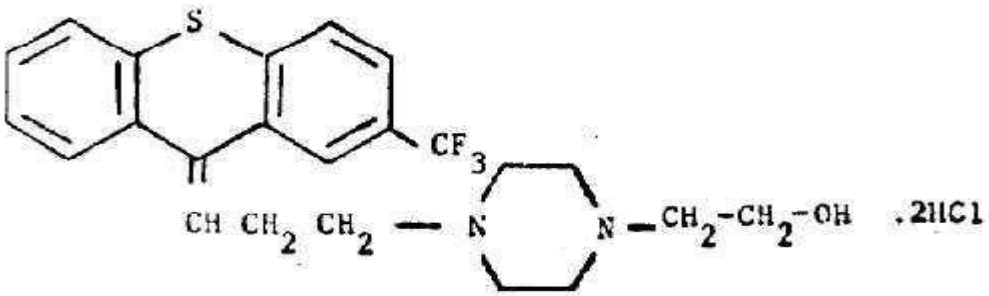
Stability of the sample, standard and reagents is required for a reasonable time to generate reproducible and reliable results. For example, 24 h stability is desired for solutions and reagents that need to be prepared for each analysis.

System suitability test provide the added assurance that on a specific occasion the method is giving, accurate and precise results. System suitability test are run everytime a method is used either before or during analysis. The results of each system suitability test are compared with defined acceptance criteria and if they pass, the Method is deemed satisfactory on that occasion.

DRUG PROFILE[20]

Flupentixol dihydrochloride is an antipsychotic neuroleptic drug. It is a thioxanthene, and therefore closely related to the phenothiazines. It is a powerful antagonist of both D_1 and D_2 dopamine receptors, and an alpha-adrenergic receptor antagonist. It's antipsychotic activity is thought to be related to blocks postsynaptic dopamine receptors in the CNS.

Molecular Structure



Nomenclature : (EZ)-2-[4-[3-[2-(trifluoromethyl) thioxanthen-9- ylidene]propyl] piperazin-1-yl] ethanol.dihydrochloride.

Molecular formula: $C_{23}H_{25}F_3N_2OS.2HCl$Molecular weight : 507.45 g / mol.

Characteristics: Off-white granular powder.

Solubility: Soluble in water, ethanol and methanol, slightly soluble in chloroform and insoluble in ether.

Category: Antipsychotic.

CHAPTER 2

OBJECTIVES

Flupentixol dihydrochloride is an antipsychotic neuroleptic drug. It is a thioxanthene, and therefore closely related to the phenothiazines. It is a powerful antagonist of both D_1 and D_2 dopamine receptors, and an alpha-adrenergic receptor antagonist. Its antipsychotic activity is thought to be related to blocks postsynaptic dopamine receptors in the CNS.

Drug analysis plays an important role in the development of drugs, their manufacture and therapeutic use. The therapeutic importance of this compound justifies research to establish analytical methods for its determination in bulk and pharmaceutical formulations.

Extensive literature survey reveals that very few analytical methods have been reported for

the estimation of Flupentixol dihydrochloride includes HPLC, HPTLC, LC-MS and UV/Visible spectrophotometry. But there are no reported spectrophotometric methods such as derivative spectroscopy, HPLC and HPTLC etc., individually for this compound.

Hence an attempt has been made to develop simple, accurate, sensitive, rapid and economic method for effective quantitative determination of Flupentixol dihydrochloride as an active pharmaceutical ingredient as well as in pharmaceutical preparations using UV/Visible spectrophotometry, High Performance Liquid Chromatography and High Performance Thin Layer Chromatography techniques.

Validation of the method was done in accordance with USP and ICH guideline for the assay of active ingredient. The methods were validated for parameters like accuracy, linearity, precision, limit of detection, limit of quantitation, ruggedness and robustness. These methods provide means to separate the components characterizeand quantify the components.

These proposed methods are suitable for the pharmaceutical analysis in analyticallaboratories. In summary, the primary objective of proposed work was to.

- Develop new, simple, sensitive, accurate, and economical analytical methods for the estimation of Flupentixol dihydrochloride.
- Validate the proposed methods in accordance with USP and ICH guidelines for the intended analytical application i.e., to apply the proposed method for analysis of this drug in their dosage form.
- Review of the literature reveals that, various methods were conducted for Flupentixol dihydrochloride alone or with other drugs. So, some new Sensitive RP-HPLC, HPTLC and Spectrophotometric methods were developed for estimation of Flupentixol dihydrochloride in bulk and pharmaceutical Formulations.

CHAPTER 3

REVIEW OF LITERATURE

1. Difference Spectrophotometric determination of some pharmaceutically important Thioxanthene derivatives in dosage forms.[21]

Fatma Aly A.

A Spectrophotometric method is described for the rapid determination of four thioxanthene derivatives, namely chlorprothixene, thiothixene, flupenthixol hydrochloride and clopenthixol hydrochloride. The method is based on measuring the first derivative spectrum of the oxidized drug relative to a solution of the underivatized drug. At the wavelength of maximum difference in first derivative (range 285–315 nm), only the oxidation products have an appreciable difference in first derivative and the oxidizing agent has no absorbance. The oxidation products (assumed to be the thioxanthone sulphoxides) are formed rapidly at room temperature by the addition of peroxyacetic acid, prepared by the slow reaction of hydrogen peroxide and glacial acetic acid. The first derivative of the absorbance is proportional to the concentration of the drugs in solution over the ranges 2–14 µ g / ml forchloroprothixene, 2–20 µ g / ml for clopenthixol hydrochloride, 2–24 µ g / ml for thiothixene and 4–40 µ g / ml for flupenthixol hydrochloride.

2. Quantification of the Antipsychotics Flupentixol and Haloperidol in humanserum by HPLC with ultraviolet detection.[22]

Walter S *et al.*

A high-performance liquid chromatography method was developed for quantification of both isomers of the Thioxanthene neuroleptic Flupentixol and of the Butyrophenone derivative Haloperidol in human serum. After extraction with diethyl ether-n-heptane (50:50, v/v), an isocratic normal-phase HPLC system with a Hypersil cyanopropyl silica column (250x4.6 mm, 5 µ g particle size) was used with ultraviolet detection at 254 nm and elution with a mixture of 920 ml acetonitrile, 110 ml of methanol, 30 ml 0.1 M ammonium acetate, and 50 ml of triethylamine.

3. Simultaneous determination of four Antipsychotic drugs in plasma by High Performance Liquid Chromatography.[23]

Garay Garcia L *et al.*

A specific reversed phase-high pressure liquid chromatography method has been developed for the simultaneous determination of Clozapine, Loxapine, Zuclopenthixol and Flupenthixol in plasma. Carpipramine, a dihydrodibenzazepine, was used as an internal standard. A liquid–liquid procedure was used to extract the drugs from human plasma. The analysis was performed on a XTerra™ MS C 18 column with UV detection. Calibration curves were linear in the range 50–1000 µg/l.

4. Quantitative determination of forty-eight Antidepressants and Antipsychotics in

human serum by HPLC Tandem Mass Spectrometry.[24]

Kirchherr H *et al.*

This method describes the simultaneous determination of Amisulpride, Amitriptyline, Aripiprazole, Benperidol, Chlorpromazine, Chlorprothixene, Citalopram, Clomipramine, Clozapine, Desipramine, Doxepin, Fluoxetine, Flupentixol, Fluphenazine, Fluvoxamine, Haloperidol, Hydroxyrisperidone, Imipramine, Levomepromazine, Maprotiline, Mianserine, Mirtazapine, Moclobemide, Norclomipramine, Nordoxepin, Norfluoxetine, Nortriptyline, O-desmethylvenlafaxine,Olanzapine, Opipramol, Paroxetine, Perazine, Perphenazine, Pimozide, Pipamperone, Quetiapine, Reboxetine, Risperidone, Sertraline, Sulpiride, Thioridazine, Trazodone, Trimipramine, Venlafaxine, Viloxazine, Ziprasidone, Zotepine and Zuclopenthixol with a single sample/triple injection approach. Drugs were assigned to subgroups covering low, medium and high concentrations (overall range of therapeutic levels to be considered: 0.5–2000 ng / ml) by further dilution of the supernatant obtained after the first protein precipitation. Chromatographicseparation was necessary for isobaric mass fragments and performed on a monolithic C18 column (50 mm × 4.6 mm) with methanol gradient and 5 mM acetate buffer at pH 3.9. The injection interval was 8 min. After electrospray ionization positive ion fragments were detected in the multiple reaction monitoring modes with an API 4000 tandem mass spectrometer.

5. Estimation of Flupentixol HCl in single dosage form by RP-HPLC method.[25]

Sheikh IA *et al.*

A simple, selective, rapid, precise and economical RP- HPLC method has been developed for estimation of *Flupenthixol* Hydrochloride in pharmaceutical dosage forms. The method was carried out on a Eurospher C-18 (250 cm× 4 mm) column with precolumn and a mobile phase consisting of Acetonitrile: Water (pH 3.0), (50:50 v / v) at a flow rate of 1 ml / min. Detection was carried out at 229nm.

6. Determination of the lipophilicity of some Psychotropic drugs by RP-TLC.[26]

Anna Hawry *et al.*

Psychotropic drugs have been chromatographed on RP-18-HPTLC plates with mobilephases containing water, an organic modifier (methanol, dioxane, acetone, acetonitrile or tetrahydrofuran), and ion-pair reagents or ammonia. R_F was measured for different concentrations of organic modifier. Relationships between solute retention and modifier

concentration were described by the Soczewiński-Wachtmeister and Schoenmaker equations.

7. The identification of Tricyclic Neuroleptics and Sulphonamides by high-performance thin-layer chromatography.[27]

Li Wan Po A *et al.*

Circular high-performance thin-layer chromatography has been used to differentiate a series of twelve Tricyclic Neuroleptics, using both normal phase and reverse phase procedures. The use of normal phase systems also allows the resolution of geometric isomers of Chlorprothixene, Clopenthixol and Flupenthixol. Thirteen Sulphonamides and Trirnethoprim may also be distinguished using HPTLC.

8. Quantitation of Seven Low-Dosage Antipsychotic Drugs in Human Postmortem Blood Using LC-MS-MS.[28]

Roman M *et al.*

In forensic toxicology, antipsychotic drugs are of considerable interest because of theirabuse potential and their involvement in intoxications and suicides. In recent years, several new drugs dosed at low levels have entered the market and have put further demands on assays used. The aim of this work was to develop a validated liquid chromatography-tandem mass spectrometry assay for the quantitation of the low- dosage antipsychotic drugs Buspirone, Fluphenazine, Flupenthixol, Perphenazine, Risperidone, Ziprasidone, and Zuclopenthixol in human postmortem blood. After liquid-liquid extraction using methyl t-butyl ether, compounds were separated on a Zorbax SB-CN column. Calibration curves were linear in the range 0.8-100 µ g / l ($r > 0.998$) for all drugs.

9. Validation of a sensitive LC-MS-MS method for simultaneous quantitation of Flupentixol and Melitracen in human plasma.[29]

Jinjing Che *et al.*

A sensitive method has been developed and validated, using LC/ESI-MS/MS, for simultaneous quantitation of Flupentixol and Melitracen Antidepressant drugs, in human plasma. The quantitation of the target compounds was determined in a positive ion mode and multiple reaction monitoring. The method involved a repeated liquid– liquid extraction with diethyl ether and analytes were chromatographed on a C-8 chromatographic column by elution with acetonitrile: water: formic acid (36:64:1, v/v/v) and analyzed by tandem mass spectrometry. The method was validated over the concentration ranges of 26.1–

2090 pg / ml for flupentixol and 0.206–4120 ng / ml for melitracen.

10. LC-MS-MS analysis of the Neuroleptics Clozapine, Flupentixol, Haloperidol, Penfluridol, Thioridazine, and Zuclopenthixol in hair obtained from Psychiatric patients.[30]

Weinmann W *et al.* Hair samples of psychiatric patients were analysed by liquid chromatography-tandem mass spectrometry (LC-MS-MS) for the Neuroleptics Clozapine, Flupentixol, Haloperidol, Penfluridol, Thioridazine, and Zuclopenthixol. In the study, these neuroleptics were administered to the patients regularly for a minimum of 6 months. Sample preparation was performed by washing, powdering with a ball mill, and extraction of drugs from hair by ultra-sonication with methanol, cleanup by solid- phase extraction and subsequent LC-MS-MS analysis using multiple reaction monitoring (MRM). Calibration was performed for all drugs in the range of 0.05 to 10ng/mg using spiked hair powder and doxepin-d3 as internal standard. 20-50 mg of hair powder was used and the detection limits of LC-MS-MS were below 0.05ng/mg for all drugs tested. Therapeutic dosage, number of subjects, hair colour and detected amounts of drugs were as follows: Clozapine (150-400 mg / day; n = 3, light brown, medium brown, black; 0.47-0.92 ng / mg), Haloperidol (150mg / 3 weeks; n = 1, black/gray; 12.2 ng / mg), Penfluridol (20-30 mg / week; n = 2, medium brown, black; 0.08 ng / mg; not detected in one case), Thioridazine (100-400 mg / day; n = 4, light brown, medium brown, black; 0.33-9.91 ng / mg, not detected in one case). Besides the active drugs also the desmethyl-metabolites of Clozapine and Thioridazine were detected by LC-MS-MS. However, Flupentixol (5 mg / day; light brown hair) and Zuclopenthixol (350 mg / 3 weeks; light brown hair) were not detected by these methods in one case each, although the drugs were administered regularly to these patients.

CHAPTER 4

METHODOLOGY

PART A: UV SPECTROSCOPY

METHOD A: ZERO ORDER DERIVATIVE SPECTROSCOPY

Selection of analytical wavelength

Appropriate dilutions were prepared for drug from the standard stock solution and the solutions were scanned in the wavelength range of 200-400 nm. The absorption spectra thus

obtained were derivatized from Zero to Second order. The first and second order derivative spectrum was selected for the analysis of the drugs.

Preparation of stock solutions

Standard Flupentixol dihydrochloride 100 mg was weighed and transferred to a 100 ml volumetric flask and dissolved in distilled water. The flask was shaken and volume was made up to the mark with distilled water to give a solution containing 1000 mcg / ml. From this stock solution, pipetted out 10 ml and placed into 100 ml volumetric flask. The volume was made up to mark with distilled water to give a solutioncontaining 100 mcg / ml.

Selection of analytical concentration ranges

From the standard stock solution of Flupentixol dihydrochloride, appropriate aliquots were pipetted out in to 10 ml volumetric flasks and dilutions were made with distilled water to obtain working standard solutions of concentrations from 3 to 100 mcg / ml.Absorbance for these solutions were measured at 229 nm. For the standard solution analytical concentration range were found to be 3-15 mcg / ml and those values were Reported in **Table: 5.1.**

Calibration curve for the Flupentixol dihydrochloride (3 - 15 mcg / ml): Appropriate volume of aliquots from standard Flupentixol dihydrochloride stock solutions were transferred to different volumetric flasks of 10 ml capacity. The volume was adjusted to the mark with distilled water to obtain concentrations of 3, 6, 9,12 and15 mcg / ml. Absorbance spectra of each solution against distilled water as blank were measured at 229 nm and the graphs o f absorbance against concentrat io n was plotted and are shown in **Fig: 5.1.** The regression equation and correlation coefficient was determined and are presented in **Table: 5.2.**

Sample preparation for determination of Flupentixol dihydrochloride fromdosage form.

Twenty tablets (fluanxol) were weighed and finely powdered. The powder equivalent to 10 mg of Flupentixol dihydrochloride was accurately weighed and transferred to volumetric flask of 100 ml capacity containing 25 ml of the distilled water andsonicated for 30 min. The flask was shaken and volume was made up to the mark with distilled water to give a solution of 100 mcg / ml. Carefully filtered through Whatmann filter paper (No. 41) and used for the estimation of Flupentixol dihydrochloride.

Validation of spectrophotometric method

Linearity and Range

The linearity of analytical method is its ability to elicit test results that are directly proportional to the concentration of analyte in sample with in a given range and wasgiven in **Fig: 5.2.** The range of analytical method is the interval between the upper andlower levels of analyte that have been demonstrated to be determined within a suitable level of precision, accuracy and linearity.

Precision

The precision of an analytical method is the degree of agreement among individual test results, when the method is applied repeatedly to multiple samplings of homogenous samples. It provides an indication of random error results and was expressed as coefficient of variation (CV).

Intra and inter-day precision

Variations of results within the same day (intra -day), variation of results between days(inter -day) were analyzed. Intra-day precision was determined by analyzing Flupentixol dihydrochloride for six times in the same day at 229 nm. Inter-day precision was determined by analyzing daily once for six days at 229 nm and % CV was calculated and were shown in **Table: 5.3.**

Accuracy

Accuracy is the closeness of the test results obtained by the method to the true value. To study the accuracy, 20 tablets were weighed and powdered and analysis of the same was carried out. Recovery studies were carried out by adding known amount of standard drug solution (9 or 10 mcg / ml) to the sample solution. The % recovery was calculated and reported in **Table: 5.4.**

Ruggedness

The solutions were prepared and analyzed with change in the analytical conditions like different laboratory conditions and different analyst and reported in **Table: 5.5.**

METHOD B: FIRST ORDER DERIVATIVE SPECTROSCOPY

In First order derivative method, the absorption maxima shows at 222 nm in the concentration range of 3-15 mcg / ml. All the procedures and parameters were same as

described in Method A. All the results are presented in **Table: 5.6** to **5.10.**

METHOD C: SECOND ORDER DERIVATIVE SPECTROSCOPY

In Second order derivative method, the absorption maxima shows at 214 nm in the concentration range of 3-15 mcg / ml. All the procedures and parameters were same as described in Method A. All the results are presented in **Table: 5.11.** to **5.15.**

PART B: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

In the present investigation, we have developed a simple and sensitive RP-HPLC method for quantitative estimation of Flupentixol dihydrochloride in bulk drug and pharmaceutical formulations.

Experimental

Instrumentation

An isocratic high performance liquid chromatography (SHIMADZU, HPLC) with LC-10ATVP, HPLC-pump K-501, with software N4000 version 1.7 and UV/Vis detector SPD-10A (SHIMADZU) was used. Phenomenex-Gemini C-18 Column was used (250 x 4.6 mm, 5 μm).

Reagents

The reference standard Flupentixol dihydrochloride was kindly gifted by Micro Laboratories Limited (Hosur, Chennai and India). The standard drugs were used without further purification. Water, Methanol and other reagents of HPLC grade was procured from Qualigens, Mumbai were used throughout the experiment. The mobile phase consists of mixture of Methanol and Water in the ratio of 65: 35 (v / v).

Preparation of working stock solution of Flupentixol dihydrochloride

About 25 mg of Flupentixol dihydrochloride was weighed accurately and dissolved in 50 ml of Water in a 100 ml volumetric flask and diluted up to the mark with water to get the concentration of 300 mcg / ml. From this, pipetted out 4 ml of the above stock solution into a 10 ml volumetric flask and diluted up to the mark with the mobile phase to get concentration 100 mcg / ml was prepared for RP-HPLC method. Resultant solution was filtered through Whatman filter paper.

Chromatographic conditions

Flow rate: 1.0 ml / min

Column: Phenomenex-Gemini C-18 (250 x 4.6 mm, 5 µm)

Detector wavelength: 229 nm

Column temperature: Ambient

Injection volume: 20 µl

Run time: 6 min.

Assay procedure

Working standard solutions containing 10 to 50 mcg / ml of Flupentixol dihydrochloride were prepared by appropriate dilution of the stock solution with the mobile phase. Twenty µ l aliquot of each solution was injected into the column for five times and the chromatograms were recorded and are presented in **Fig: 5.7**. The retention time was found to be 4.28 min. Calibration graph was constructed by plotting the mean peak area as a function of Flupentixol dihydrochloride concentration.

Analysis of formulation

Twenty tablets (Fluanxol) were accurately weighed and finely powdered. Tablet powder equivalent to 25 mg of Flupentixol dihydrochloride was weighed accurately and dissolved in 50 ml of Water in a 100 ml volumetric flask and diluted up to the mark with water to get the concentration of 250 mcg / ml. From this, pipetted out 4 ml of the above stock solution into a 10 ml volumetric flask and diluted up to the mark with the mobile phase to get concentration 100 mcg / ml was prepared for RP-HPLC method. Resultant solution was filtered through Whatman filter paper. The final solution was injected into chromatographic system for three times.

VALIDATION OF ANALYTICAL METHOD

Validation of an analytical method is the process to establish by laboratory studies that the performance characteristic of the method meets the requirements for the intended analytical application. Performance characteristics are expressed in terms of analytical parameters.

1. Accuracy

The accuracy of a method was inferred by establishing the precision and linearity of the standards and given in **Table: 5.17.**

2. Precision

The precision of the method was demonstrated by inter-day and intra-day variation studies. In the intra-day studies, six repeated injections of standard solution was made and the response factor of drug peak and % RSD were calculated and present in **Table: 5.18.** The chromatogram was shown in **Fig: 5.8**. In the inter-day variationstudies, six repeated injections of standard solution were made for six consecutivedays and response factor of drugs peak and % RSD were calculated shown in **Table: 5.18.** From the data obtained, the developed method was found to be precise.

3. Linearity

The linearity of the method was demonstrated over the concentration range of 10- 50 mcg / ml of the target concentration. Aliquots of 10, 20, 30, 40 and 50 mcg / ml were prepared from above prepared stock solution. Different concentrations of the pure drugwere injected into the chromatographic system. Calibration curve of Flupentixol dihydrochloride was constructed by plotting peak area vs. applied concentration of Flupentixol dihydrochloride. The obtained results shown an excellent correlation between peak area and concentration of pure drug within the concentration range & it has shown in **Fig: 5.9.** The correlation coefficient for the average area at each level versus concentration of analyte was calculated and is presented in **Table: 5.19.** and their calibration parameters were shown in **Table: 5.20.**

Standard deviation (SD) = $\sigma = \sqrt{\frac{\Sigma(x-x)^2}{n-1^1}}$

Where, x = sample

x_1 = mean value of samplesn = number of samples.

The correlation Coefficient and Percentage curve fittings were calculated by using the following formula.

$R = \sum (X-X)(Y-Y) / (n-1) Sx Sy$

Where, X = Concentration

Y = Instrumental response

Sx = Standard deviation of x

Sy = Standard deviation of y

Percentage Curve Fitting = 100 X Correlation Coefficient.

4. Limit of detection (LOD)

Limit of detection is determined by the analysis of samples with known concentrations of

analyte and by establishing the minimum level at which the analyte can be reliably detected. From the standard stock solution 4 ml was pipetted out into 10 ml volumetric flask and the volume was made up to the mark with mobile phase. From this solution, Pipettedout 1ml of 100 mcg / ml solution into a 10 ml of volumetric flask and dilute up to the mark with diluent. Final dilution was made by pipetting 0.11 ml (10 mcg / ml) of above solution into a 10 ml of volumetric flask and diluted up to the mark with diluent. The solution was injected for three times and chromatogram was shown in **Fig: 5.10.** The Limit of detection was found to be 0.11 mcg / ml for Flupentixol dihydrochloride. The results of LOD were shown in **Table: 5.22.**

5. Limit of quantitation (LOQ)

Based on the LOD strength (0.11 mcg / ml, standard solution), the LOQ values were calculated by multiplication with three times.

From the standard stock solution 4 ml was pipetted out was placed into 10 ml volumetric flask and volume was made up to the mark with mobile phase. Pipetted out1ml of 100 mcg / ml solution into a 10 ml of volumetric flask and diluted up to the mark with diluent. Further pipetted 0.34 ml of above diluted solution into a 10 ml of volumetric flask and dilute up to the mark with diluent. The solution was injected for three times and chromatogram was shown in **Fig: 5.11.** The Limit of quantitation wasfound to be 0.34 mcg / ml for Flupentixol dihydrochloride. The results of LOQ were Shown in **Table: 5.23.**

6. Ruggedness

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

Flupentixol dihydrochloride sample equivalent to 25 mg was weighed and dissolved in a 100 ml volumetric flask containing mobile phase(50 ml), sonicated for few mints and the final volume was made with mobile phase. The samples were injected into the column, chromatogram was recorded and was shown in **Fig: 5.12.** The results of ruggedness were shown in the **Table: 5.24.**

7. Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. For the determination of robustness, a number of method parameters such as pH, flow rate, column temperature, injection volume, detection wavelength, or mobile phase composition, are varied within a realistic range, and the quantitative influence of the variables is determined. Thesample was analyzed separately by slightly changes in the analytical method as given below. By changing the flow rate of mobile phase to 1.0 ± 0.2 ml / min. The chromatograms were recorded and were shown in **Fig: 5.13. and 5.14.** The retention time values were measured. The robustness results were shown in **Table: 5.26. and 5.27**.

By changing ratio of the mobile phase i.e. Methanol: Water from 70: 30 to 60: 40. The chromatograms were recorded and were shown in **Fig: 5.15. and 5.16**. The retention time values were measured. The robustness results were shown in **Table:** 5.29**.** and 5.30.

8. System suitability

System-suitability tests are an integral part of method development and are used to ensure adequate performance of the chromatographic system. Retention time (R_t), number of theoretical plates (N) and tailing factor (T) were evaluated for six replicate injections of the drug at a concentration of 100 mcg / ml. The results given in **Table: 5.21.** were within acceptable limits.

PART C: HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

HPTLC (High Performance Thin Layer Chromatography) is the most simple separation technique today available to the analyst. It can be considered a time machine that can speed your work and allows you to do many things at a time usually not possible with other analytical techniques.

EXPERIMENTAL

Instrumentation

A LINOMAT 5-HPTLC with CAMAG- TLC Scanner 3, Win-CAT software, version 1.44 was used.

Reagents and pharmaceutical preparations

An analytically pure sample of Flupentixol dihydrochloride was procured as gift sample from

Micro laboratories (Hosur, Chennai, India). All chemicals including Glacial Acetic Acid and Toluene were of analytical grade and were used throughoutthe experiment.

Standard Solutions

Standard Flupentixol dihydrochloride 25 mg was weighed and transferred to a 50 ml volumetric flask and dissolved in methanol. The solution was sonicated for 30 min and volume was made up to the mark with methanol to give a solution containing 500 mcg / ml. Appropriate volume of aliquots from standard Flupentixol dihydrochloride stock solutions were prepared by diluting the stock solution to give a series of spots covering the range 300 to 1500 ng of Flupentixol dihydrochloride.

Sample Preparation

Twenty tablets were weighed and the average weight was calculated. The Tablets were crushed to furnish a homogeneous powder and a quantity equivalent to 25 mg of Flupentixol dihydrochloride was transferred to a 50 ml calibrated volumetric flask. The powder dissolved in methanol. That solution was then sonicated for 30 min. Then cooled to room temperature and diluted with methanol. The solution was filtered through Whatman No. 41 filter paper and the filtrate was used as sample solution.

Chromatography Conditions

Stationary phase: Pre coated silica gel 60 F_{254} on aluminium sheets

Mobile phase: Toluene: Glacial acetic acid (7:3, v / v)

Chamber saturation: 20 minutes

Migration distance: 60 mm

Band widthL: 8 mm

Slit dimensions: 6 × 0.45 mm

Source of radiation: Deuterium lamp

Scanning wavelength: 229 nm

R_F value: 0.21 ± 0.02

Development time: 20 min

VALIDATION OF THE METHOD

1. Specificity

The optimized solvent system yielded a symmetrical peak for the drug with R_F 0.21 ± 0.02 was shown in **Fig: 5.17.** A typical absorption spectrum of Flupentixol dihydrochloride is

shown in **Fig: 5.18.** The wavelength 229 nm was selected for detection because it has better detection sensitivity for the drug. The spot for Flupentixol dihydrochloride from the tablet formulation was identified by comparing its R_F value and its absorbance / reflectance spectrum with those of standardFlupentixol dihydrochloride, as shown in **Fig: 5.19.**

2. Linearity

Five different concentrations of Flupentixol dihydrochloride were prepared from stock solution in the range of 300, 600, 900, 1200 and 1500 ng / spot. Flupentixol dihydrochloride of different concentrations were applied to a prewashed HPTLC plate. The plate was developed, dried, and scanned as described above. The detector response to the different concentrations was measured. The chromatograms wereobtained and the drug peak-area was calculated for each concentration level and given in **Table: 5.31.** The drug peak-area was calculated for each concentration level and a graph was plotted of drug concentration against the peak area and shown in **Fig: 5.20.** The linearity of response for Flupentixol dihydrochloride was assessed in the concentration ranges 300 to 1500 ng / spot. The slope, intercept, and correlation coefficient were determined and shown in **Table: 5.32.** 3D of linearity studies wasshown in **Fig: 5.21.**

3. Sensitivity

The sensitivity of measurement of Flupentixol dihydrochloride by use of the proposed method was estimated in terms of the limit of quantitation (LOQ) and the lowest concentration detected under the chromatographic conditions as the limit of detection (LOD). The LOD and LOQ were calculated by the use of the equations LOD = 3.3 x N / B and LOQ = 10 x N / B where N is the standard deviation of the peak areas of the drug (n = 3), taken as a measure of the noise, and B is the slope of the corresponding calibration plot. The results of LOD and LOQ were shown in **Table: 5.32.**

4. Precision

The precision of the analytical method was studied by analysis of multiple sampling of homogeneous sample. The precision expressed as standard deviation or relative standard deviation.

System precision: Standard solution of Flupentixol dihydrochloride was prepared as per testing procedure and injected into the HPTLC system in six replicates. 3D of system precision study was given in **Fig: 5.22.** % RSD for peak area obtained in six replicate

injections was calculated and reported in **Table: 5.33** *Method precision:* The Six different determinations of Flupentixol dihydrochloride sample (from tablets) were performed as per testing procedure and chromatograms were recorded and were shown in **Fig: 5.23.** % RSD was calculated and results are presented in **Table: 5.34.**

5. Accuracy

The accuracy of the method was determined by use of standard additions at three different levels, i.e. multiple-level recovery studies. Sample stock solution of the tabletformulation at a concentration of 500 mcg / ml Flupentixol dihydrochloride wasprepared. A 50 %, 100 %, and 150 % of the standard drug solution were added to sample solution and the recovery [%] was determined. Values were found to be withinthe limits given in **Table: 5.35.** 3D of accuracy studies are presented in **Fig: 5.24.**

6. Ruggedness

Ruggedness is a measure of the reproducibility of a test result under normal, expected operating condition from instrument to instrument and from analyst to analyst. The results of ruggedness testing are reported in the **Table: 5.36.**

7. Stability studies

The sample and standard solutions were prepared freshly in order to test the stability of drugs. No decomposition of the drug as observed during chromatogram development. No decrease in the concentration of drug on the plate was observedwithin 24 h. Because a decrease in the amount of Flupentixol dihydrochloride was observed 24 h after development, chromatograms were scanned within 1 h of development.3D of stability studies given in **Fig: 5.25.**

CHAPTER 5

RESULTS

PART A: UV SPECTROSCOPY

METHOD A: ZERO ORDER DERIVATIVE SPECTROSCOPY

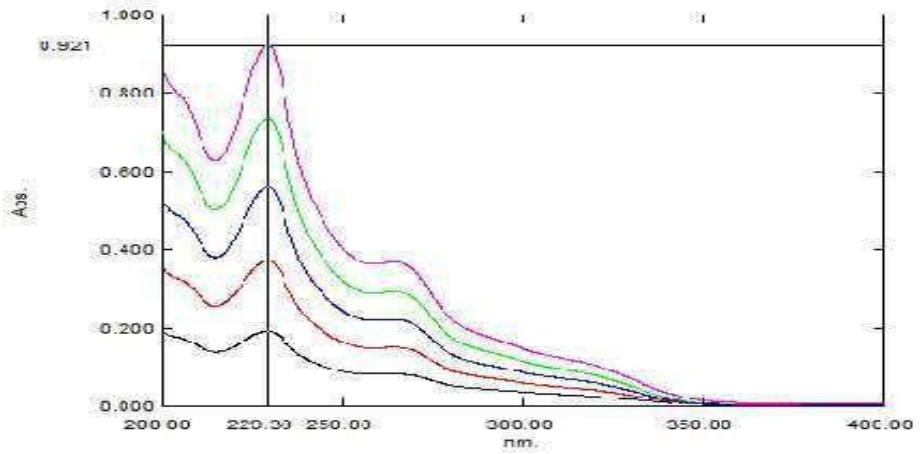


Fig: 5.1. Zero order spectra of Flupentixol dihydrochloride at 229 nm.

Table: 5.1: Results of calibration curve at 229 nm for Flupentixol dihydrochloride by Zero order derivative spectroscopy.

Sl. no.	Conc. (mcg / ml)	Absorbance at 229 nm
1	3	0.192
2	6	0.372
3	9	0.560
4	12	0.735
5	15	0.921

Table: 5.2: Optimum conditions, Optical characteristics and Statistical data of the Regression equation in UV method.

Parameters	UV Method
λmax (nm)	229
Beer's law limits (mcg / ml)	3-15
Molar extinction coefficient (L.mol-1 cm-1)	0.062 X 104
Sandell's sensitivity (mcg/cm2 0.001 absorbance units) -	0.015986
Regression equation (Y*)	Y = 0.0612C + 0.0046
Slope (b)	0.0612
Intercept (a)	0.0046
Correlation coefficient(r2)	0.9999
Intraday Precision (% RSD**) Inter day Precision (% RSD**)	0.27 0.20
Limit of detection (mcg / ml)	0.128
Limit of quantitation (mcg / ml)	0.390

*Y= b C + a where C is the concentration of Flupentixol dihydrochloride inmcg / ml and Y is the absorbance at the respective λ_{max}.

**Average of six determinations.

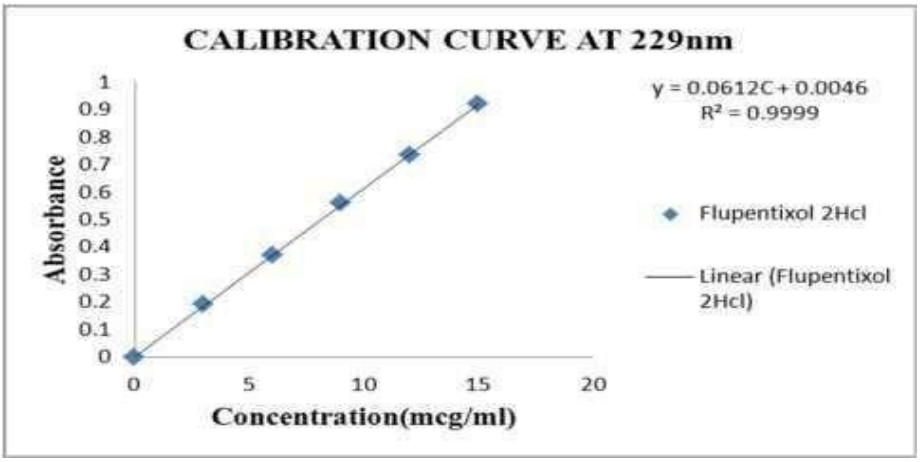


Fig: 5.2. Linearity curve for Flupentixol dihydrochloride at 229 nm by Zero order derivative spectroscopy.

Table: 5.3: Determination of Precision for Flupentixol dihydrochloride at 229 nm by Zero order derivative spectroscopy.

Conc. mcg / ml	Intra-day Absorbance Mean ± SD**	% CV	Inter-day Absorbance Mean ± SD**	% CV
3	0.193 ± 0.001	0.518135	0.192 ± 0.001155	0.599326
6	0.372 ± 0.000577	0.155063	0.327 ± 0.001549	0.154924
9	0.561 ± 0.001528	0.272124	0.561 ± 0.001155	0.205585
12	0.738 ± 0.003	0.406504	0.737 ± 0.002517	0.341158
15	0.922 ± 0.001528	0.165555	0.922 ± 0.001155	0.125193

**Average of six determinations.

Table: 5.4: Determination of Accuracy results for Flupentixol dihydrochloride by Zero order derivative spectroscopy.

Tablet	Amount of sample (mcg / ml)	Amount of drug added (mcg / ml)	Amount Recovered (mcg / ml)	% Recovery ± SD**
Sample	9	4.5	13.43	98.63 ± 0.59
	9	9	17.88	98.71 ± 0.87
	9	13.5	22.48	99.87 ± 0.27

Table: 5.5: Ruggedness results for Flupentixol dihydrochloride at 229 nm by Zero order derivative spectroscopy.

Tablet	Label claim (mg)	Analyst I		Analyst II	
		Amount found (mg)	Recovery ± SD** (%)	Amount found (mg)	Recovery ± SD** (%)
Sample	3	2.971	99.03 ± 0.07	2.982	99.39 ± 0.03

** Average of six determinations.

METHOD B: FIRST ORDER DERIVATIVE SPECTROSCOPY

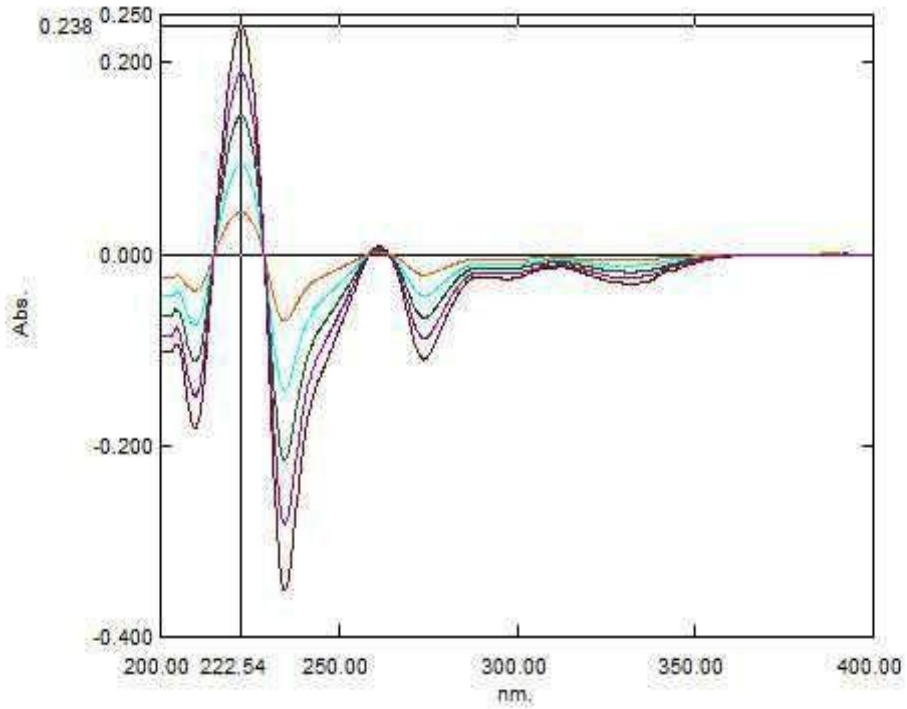


Fig: 5.3. First order spectra of Flupentixol dihydrochloride at 222 nm.

Table: 5.6: Results of calibration curve at 222 nm for Flupentixol dihydrochloride by First order derivative spectroscopy.

Sl. no.	Conc. (mcg / ml)	Absorbance at 222 nm
1	3	0.044
2	6	0.093
3	9	0.143
4	12	0.186
5	15	0.237

Table: 5.7: Optimum conditions, Optical characteristics and Statistical data of the Regression equation in First order Derivative Method.

Parameter	First order Derivative Method
λmax (nm)	222
Beer's law limits (mcg / ml)	3-15 4
Molar extinction coefficient (L.mol-1^{-1} cm)	0.016 X 10
Sandell's sensitivity	0.0625

(mcg/cm 2 -0.001 absorbance units)	
Regression equation (Y*)	Y = 0.0158C - 0.0015
Slope (b)	0.0158
Intercept (a)	-0.0015
Correlation coefficient (r^2)	0.9996
Intraday Precision (% RSD**)Inter day Precision (% RSD**)	0.401 0.402
Limit of detection (mcg / ml)	0.12
Limit of quantitation (mcg / ml)	0.37

*Y= b C + a where C is the concentration of Flupentixol dihydrochloride in mcg / ml and Y is the absorbance at the respective □max.

**Average of six determinations.

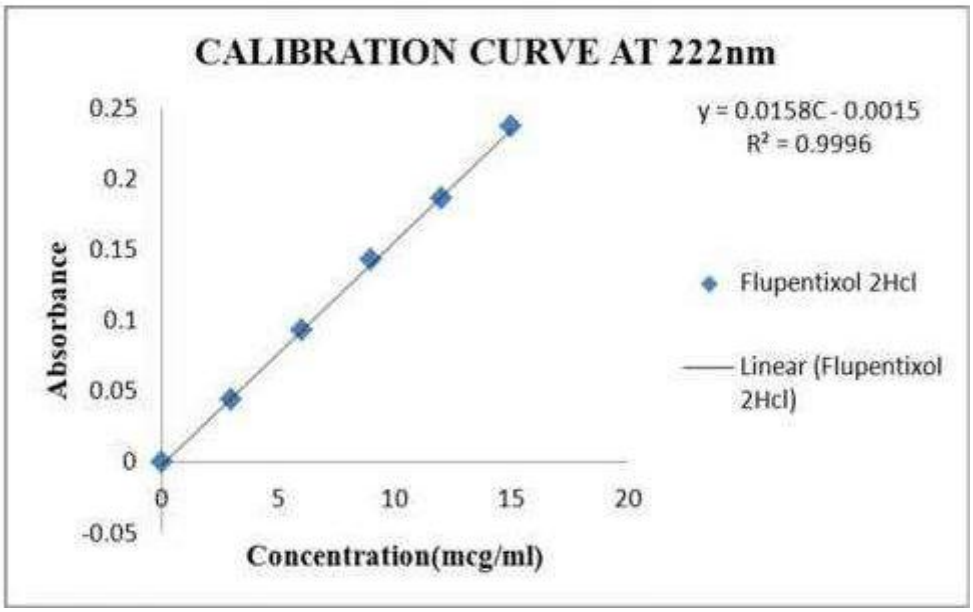


Fig: 5.4. Calibration curve for Flupentixol dihydrochloride at 222 nm by first order derivative spectroscopy.

Table: 5.8: Determination of Precision for Flupentixol dihydrochloride at 222 nm by First order derivative spectroscopy.

Conc. mcg / ml	Intra-day Absorbance Mean ± SD**	% CV	Inter-day Absorbance Mean ± SD**	% CV
3	0.044 ± 0.000577	1.302294	0.044 ± 0.000577	1.292575
6	0.094 ± 0.001528	1.619285	0.093 ± 0.000577	0.61859
9	0.143 ± 0.000577	0.401868	0.143 ± 0.000577	0.402803
12	0.187 ± 0.001732	0.92623	0.187 ± 0.001528	0.815405

15	0.239 ± 0.001732	0.724707	0.238 ± 0.001528	0.640025

**Average of six determinations.

Table: 5.9: Determination of Accuracy results for Flupentixol dihydrochloride by First order derivative spectroscopy.

Tablet	Amount of sample (mcg / ml)	Amount of drug added (mcg / ml)	Amount Recovered (mcg / ml)	Recovery ±SD**
Sample	9	4.5	13.48	99.33 ± 0.34
	9	9	17.92	99.21 ± 0.61
	9	13.5	22.58	100.87 ± 0.77

**Average of six determinations.

Table: 5.10: Ruggedness results for Flupentixol dihydrochloride at 222 nm by First orderderivative spectroscopy.

Tablet	Label claim (mg)	Analyst I		Analyst II	
		Amount found (mg)	Recovery ± SD** (%)	Amount found (mg)	Recovery ± SD** (%)
Sample	3	2.989	99.62 ± 0.08	2.984	99.45 ± 0.08

** Average of six determinations.

METHOD C: SECOND ORDER DERIVATIVE SPECTROSCOPY

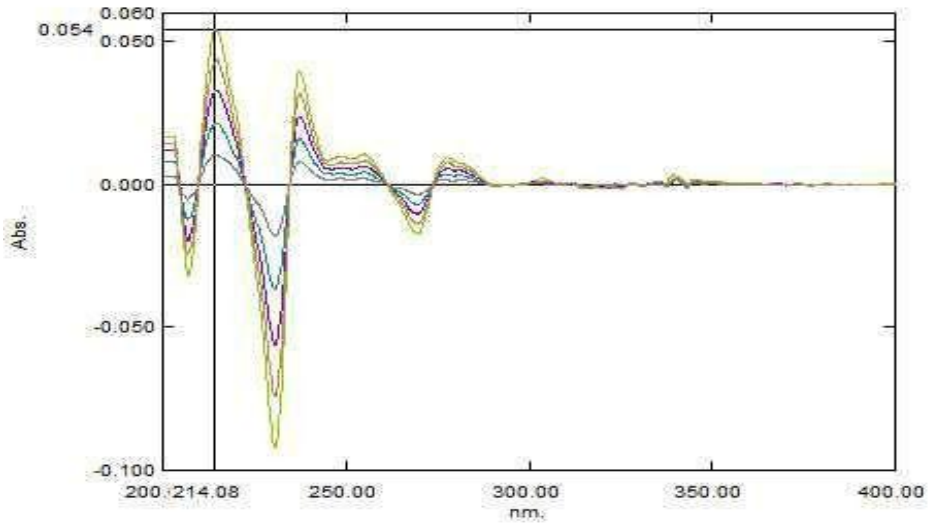


Fig: 5.5. Second order spectra of Flupentixol dihydrochloride at 214 nm.

Table: 5.11: Results of calibration curve at 214 nm for Flupentixol dihydrochloride by Second order derivative spectroscopy.

Sl. No.	Conc. (mcg / ml)	Absorbanceat 214 nm
1	3	0.011
2	6	0.022
3	9	0.032
4	12	0.043
5	15	0.054

Table: 5.12: Optimum conditions, Optical characteristics and Statistical data of the Regression equation in Second order Derivative Method.

Parameters	Second order Derivative Method
λmax (nm)	214
Beer's law limits (mcg / ml)	3-15
Molar extinction coefficient (L.mol-1 cm-1)	0.000533 X 104
Sandell's sensitivity (mcg/cm2 0.001 absorbance units)	1.875
Regression equation (Y*)	Y = 0.0036C + 0.0001
Slope (b)	0.0036
Intercept (a)	0.0001
Correlation coefficient(r2)	0.9998
Intraday Precision (% RSD**) Inter day Precision (% RSD**)	1.78 1.76
Limit of detection (mcg / ml)	0.36
Limit of quantitation (mcg / ml)	1.11

*Y= b C + a where C is the concentration of Flupentixol dihydrochloride inmcg / ml and Y is the absorbance at the respective λ_{max}.

**Average of six determinations.

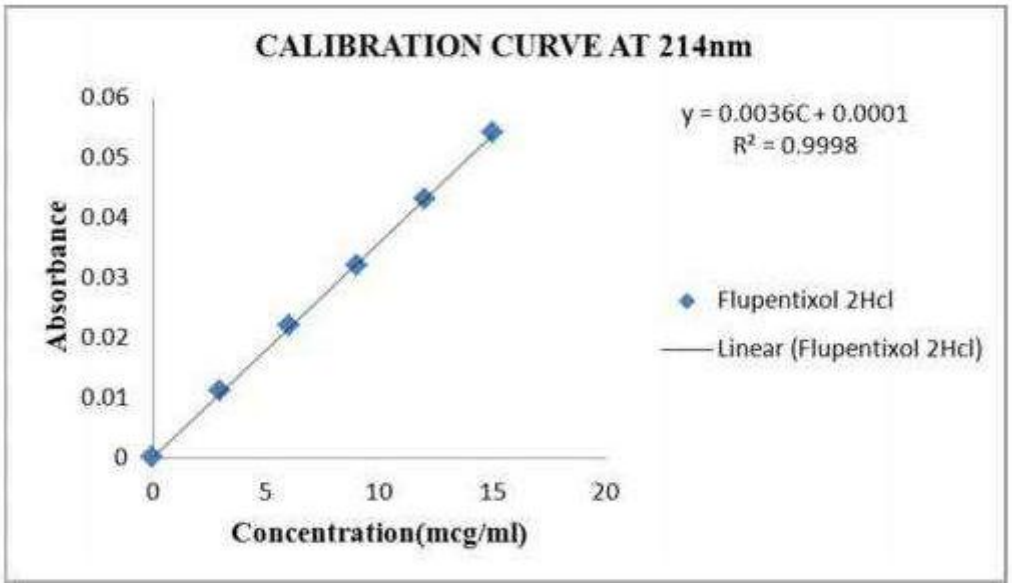


Fig: 5.6. Determination of Linearity for Flupentixol dihydrochloride at 214 nm by Second order derivative spectroscopy.

Table: 5.13: Determination of Precision for Flupentixol dihydrochloride at 214 nm by Second order derivative spectroscopy.

Conc. mcg / ml	Intra-day Absorbance Mean ± SD**	% CV	Inter-day Absorbance Mean ± SD**	% CV
3	0.010 ± 0.000577	5.587261	0.011 ± 2.12E-18	1.93E-14
6	0.021 ± 0.000577	2.706329	0.021 ± 0.000577	2.664694
9	0.032 ± 0.000577	1.785619	0.032 ± 0.000577	1.767399
12	0.043 ± 0.000577	1.332347	0.043 ± 0.000577	1.332347
15	0.054 ± 0.000577	1.062608	0.054 ± 0.000577	1.056129

**Average of six determinations.

Table: 5.14: Determination of Accuracy results for Flupentixol dihydrochloride by Secondorder derivative spectroscopy.

Tablet	Amount of sample (mcg / ml)	Amount of drug added (mcg / ml)	Amount Recovered (mcg / ml)	Recovery ±SD**
Sample	9	4.5	13.44	99.43 ± 0.51
	9	9	17.94	99.18 ± 0.27
	9	13.5	22.49	99.87 ± 0.42

**Average of six determinations.

Table 5.15: Ruggedness results for Flupentixol dihydrochloride at 214 nm by Second orderderivative spectroscopy.

Tablet	Label claim (mg)	Analyst I		Analyst II	
		Amount found (mg)	Recovery ± SD** (%)	Amount found (mg)	Recovery ± SD** (%)
Sample	3	2.988	99.75 ± 0.11	2.979	99.28 ± 0.08

** Average of six determinations.

PART B: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

In RP-HPLC method, HPLC conditions were optimized to obtain, an adequate separation of eluted compounds. Initially, various mobile phase compositions were tried to elute title ingredient. Mobile phase and flow rate selection was based on peak parameters (height, capacity, theoretical plates, tailing or symmetry factor), run time and resolution. The system with mobile phase containing Methanol: Water (65:35) with 1 ml / min flow rate was quite robust. The optimum wavelength for detection was229 nm at which better detector response for the drug was obtained. For the drug Flupentixol dihydrochloride, peak of retention time was 4.28 ± 0.10 min.

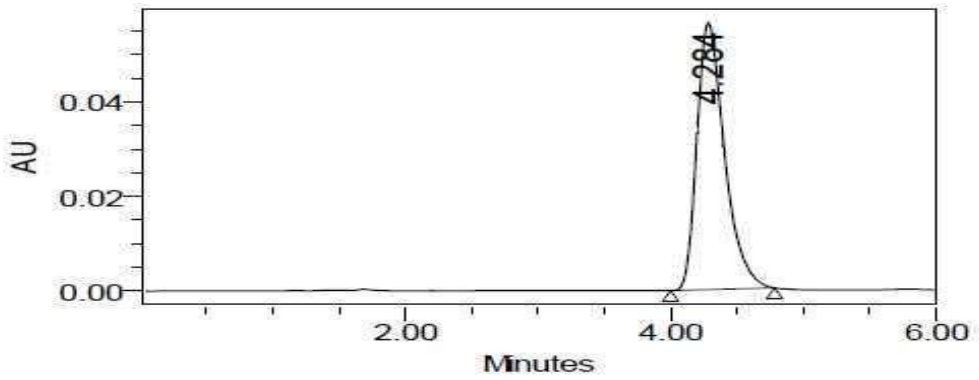


Fig: 5.7. Chromatogram of Flupentixol dihydrochloride at 229 nm.

Table: 5.16: Characteristic parameters of Flupentixol dihydrochloride for the proposed RP-HPLC method.

Parameters	RP-HPLC
Calibration range (mcg / ml)	10-50
Detection wavelength	229 nm
Mobile phase (Methanol: Water)	65:35
Retention time	4.28 ± 0.10 min
Regression equation (Y*)	Y= 26122 C+ 25166
Slope (b)	26122
Intercept (a)	25166
Correlation coefficient(r^2)	0.9996
Intraday Precision (% RSD*)Interday Precision (% RSD*)	0.25 1.62

Limit of detection (mcg / ml)	0.11
Limit of quantitation (mcg / ml)	0.34

*Y = b C + a, where X is the concentration of compound in mcg / ml and *Y* is the peak area.

VALIDATION OF ANALYTICAL METHOD

Validation of an analytical method is the process to establish by laboratory studies that the performance characteristic of the method meets the requirements for the intended analytical application. Performance characteristics were expressed in terms of analytical parameters.

1. ACCURACY

The accuracy of the method was inferred by establishing the precision and linearity studies of standard drug.

Table: 5.17: Accuracy results for Flupentixol dihydrochloride.

Sample No.	Spike Level	Amount (mcg / ml) added	Amount (mcg / ml) found	% Recovery	Mean % Recovery
1	50 %	15	14.84	98.93	98.95
	50 %	15	14.85	99.00	
	50 %	15	14.84	98.93	
2	100 %	30	29.93	99.76	99.88
	100 %	30	30.01	100.03	
	100 %	30	29.96	99.86	
3	150 %	45	44.92	99.82	99.85
	150 %	45	44.92	99.82	
	150 %	45	44.96	99.91	

2. *PRECISION*

The precision of the analytical method was studied by analysis of multiple sampling of homogeneous sample. The precision results were expressed as standard deviationor relative standard deviation.

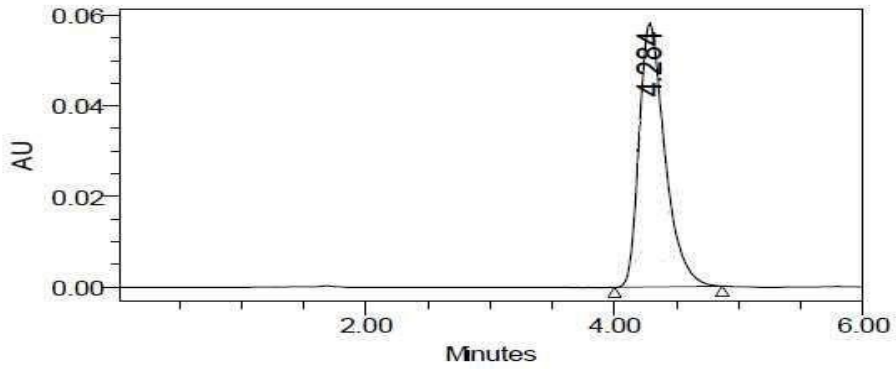


Fig 5.8: Chromatogram of Flupentixol dihydrochloride at 229 nm.

Table 5.18: Precision results for Flupentixol dihydrochloride.

Sr. No.	Concentration (mcg / ml)	Intraday precision (Area)	Interday precision (Area)
1	30	796583	748880
2	30	799542	753309
3	30	801734	739789
4	30	801228	774168
5	30	801432	756753
6	30	801438	766285
Mean		800326	756531
Std.Dev		1993.9	12292.0
%RSD.		0.25	1.62

3. *LINEARITY*

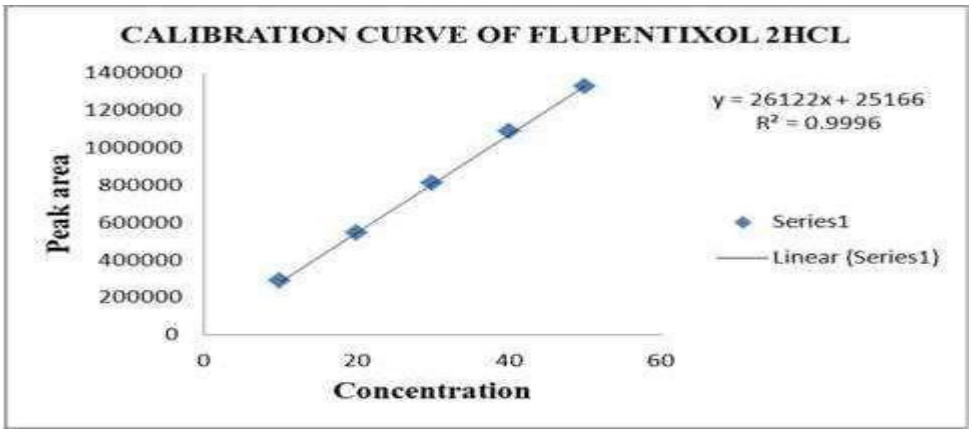


Fig 5.9: Calibration curve of Flupentixol dihydrochloride at 229 nm.

Table 5.19: Linearity results for Flupentixol dihydrochloride.

Conc.(mcg / ml)	10	20	30	40	50
Avg. Area**	287387	542641	807576	1083461	1323084
Correlation	0.9996				

** Average of five determinations.

Table 5.20: Calibration parameters of Flupentixol dihydrochloride.

Parameter	Results
Slope	26122
Intercept	25166
Correlation co-efficient	0.9996
% Percentage CurveFitting	99.96

Table 5.21: System suitability studies of Flupentixol dihydrochloride by RP-HPLC method.

Property	Values	Required limits
Retention time (R_t)	4.28 ± 0.10	RSD ≤ 1%
Theoretical plates (N)	2884.74	$N > 2000$
Tailing factor (T)	1.30	$T \leq 2$

4. *LIMIT OF DETECTION (LOD).*

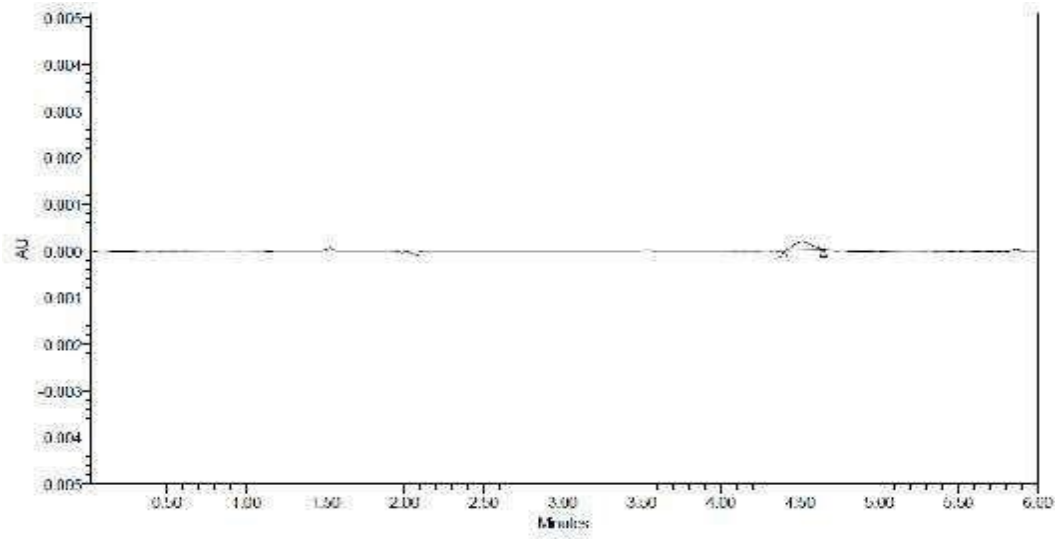


Fig 5.10: Chromatogram of Limit of detection.

Table 5.22: LOD results for Flupentixol dihydrochloride.

Injection No.	Peak Area	% RSD
1	2962	
2	2937	
3	2924	0.65

5. *LIMIT OF QUANTITATION (LOQ)*

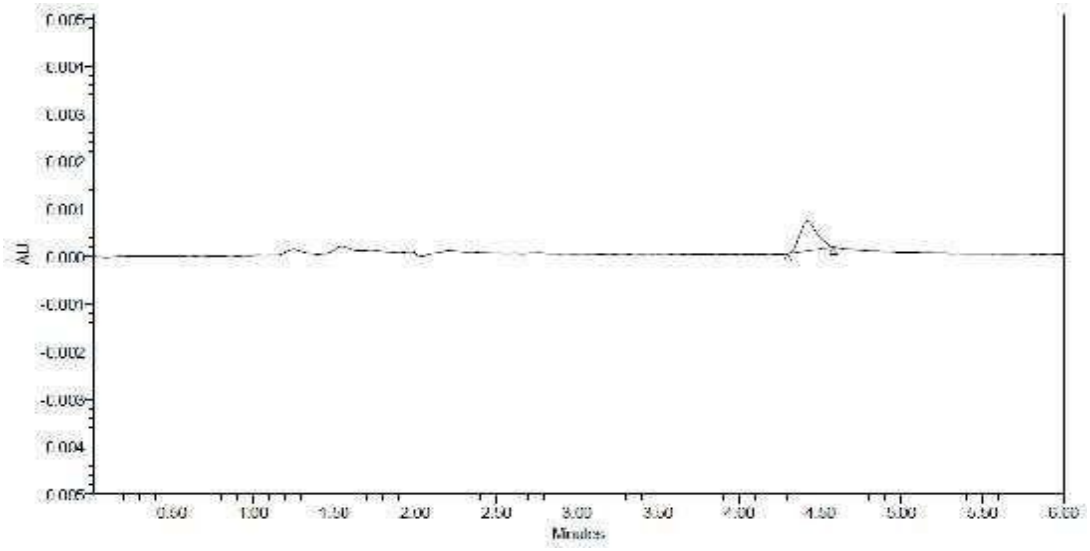


Fig 5.11: Chromatogram of Limit of Quantitation.

Table 5.23: LOQ results for Flupentixol dihydrochloride.

Injection No.	Peak Area	% RSD
1	9152	0.33
2	9213	
3	9176	

6. *RUGGEDNESS*

Ruggedness is a measure of the reproducibility of a test result under normal, expected operating condition from instrument to instrument and from analyst to analyst.

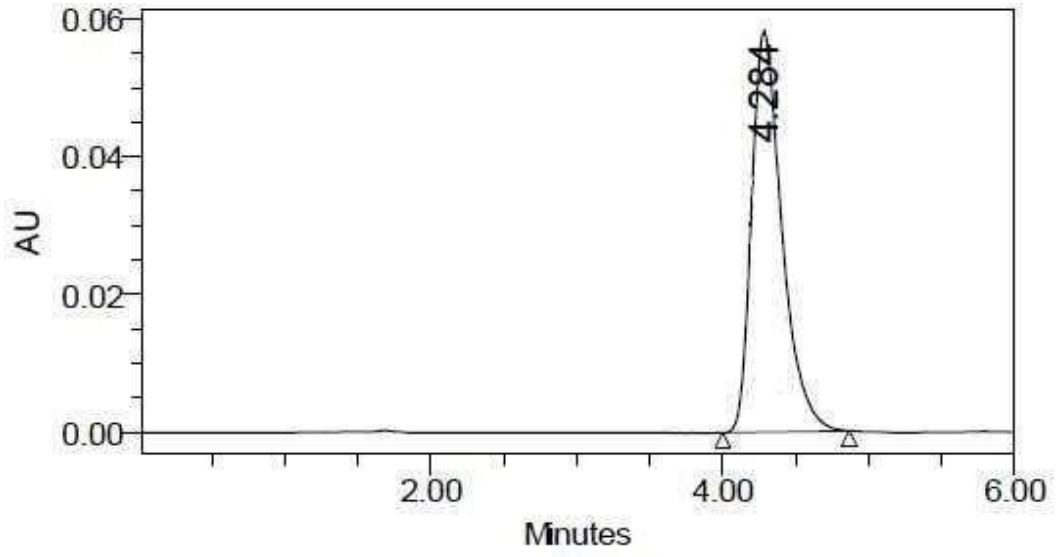


Fig: 5.12. Chromatogram of Ruggedness.

Table 5.24: Ruggedness studies of Flupentixol dihydrochloride by RP-HPLC method.

Tablet	Label claim (mg)	Analyst I		Analyst II	
		Amount found (mg)	Recovery ± SD** (%)	Amount found (mg)	Recovery ± SD** (%)
Sample	3	2.98	99.33 ± 0.11	2.97	99.04 ± 0.27

**Mean ± S.D. from six determinations

7. *ROBUSTNESS*

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

Table 5.25: (a). Chromatographic Condition: Change in flow rate

SI. No	Change in flow rate	R.T
01	0.8 ml / min	5.20
02	1.2 ml / min	3.49

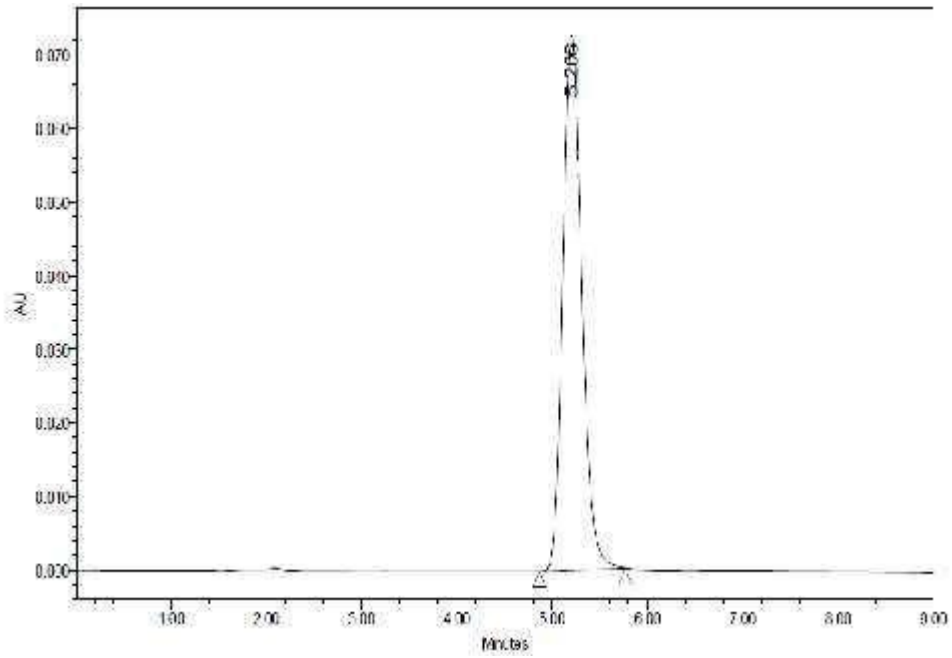


Fig 5.13: Chromatogram of Robustness (Flow rate 0.8).

Table 5.26: Robustness results for Flupentixol dihydrochloride: (Flow rate 0.8)./

RT	Peak Area	USP Plate Count	USP Tailing
5.20	1005581	3295	1.18

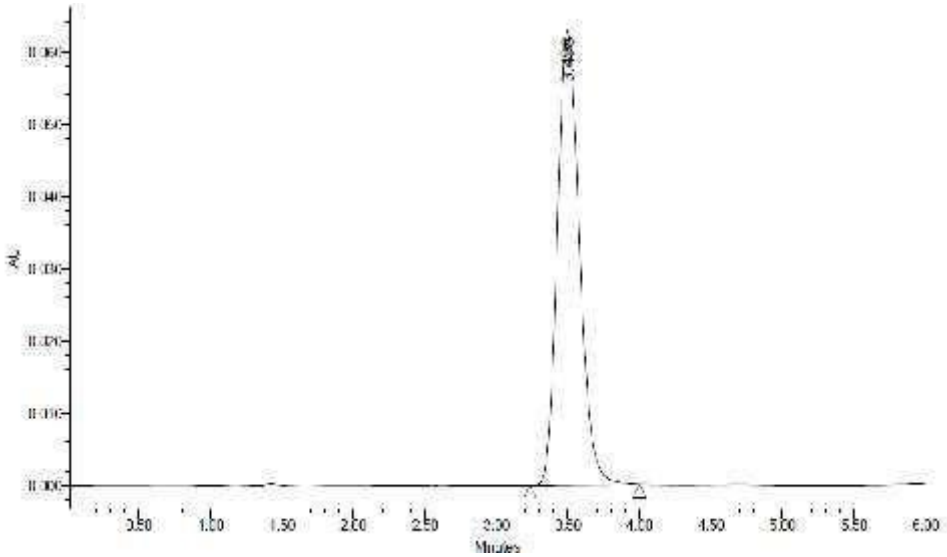


Fig 5.14: Chromatogram of Robustness (Flow rate 1.2).

Table 5.27: Robustness results for Flupentixol dihydrochloride: (Flow rate 1.2).

RT	Peak Area	USP Plate Count	USP Tailing
3.49	672811	2465	1.17

Table 5.28: (b). Chromatographic Condition: Change in mobile phase.

SI. No	Change in mobile phase	R.T
01	60: 40	5.89
02	70: 30	3.42

Mean ± S.D. from six determinations.

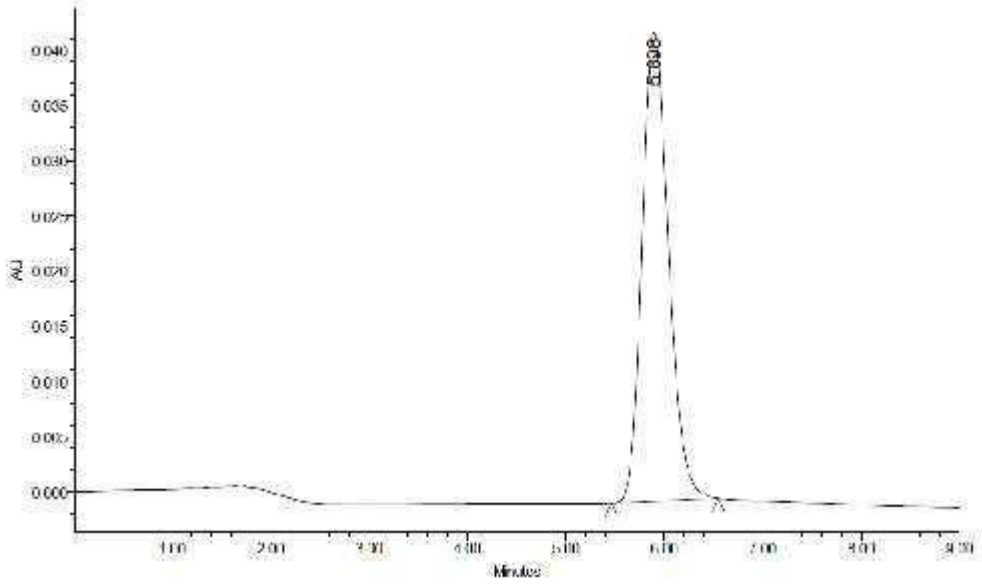


Fig: 5.15. Chromatogram of Robustness (Methanol: Water= 60:40).

Table 5.29: Robustness results for Flupentixol dihydrochloride: (Methanol: Water=

60:40).

RT	Peak Area	USP Plate Count	USP Tailing
5.89	795737	2286	1.20

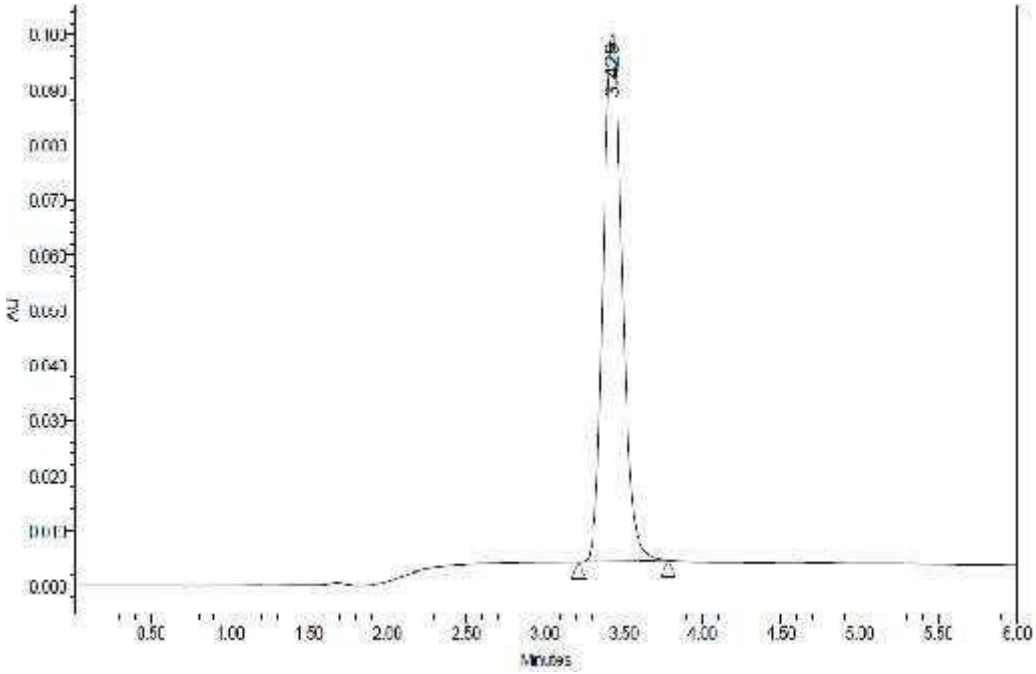


Fig 5.16: Chromatogram of Robustness (Methanol: Water= 70:30).

Table 5.30: Robustness results for Flupentixol dihydrochloride: (Methanol: Water= 70:30).

RT	Peak Area	USP Plate Count	USP Tailing
3.42	810610	3804	1.14

PART C: HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

Because the use of planar chromatography for determination of Flupentixol dihydrochloride has not been reported, we decided to investigate the feasibility of high performance thin layer chromatography (HPTLC), coupled with densitometric detection, as an alternative technique for quality control of Flupentixol dihydrochloride products. The ICH guidelines for quality assurance by planar chromatography have been adopted for validation of method specificity, accuracy and precision.

A full UV-Visible scan of Flupentixol dihydrochloride spots revealed two maxima at λ = 229 and 269 nm (Fig. 2). The 229 nm was chosen for quantification. The R_F valueof Flupentixol dihydrochloride after development with the mobile phase Toluene:Glacial acetic acid (7:

3, v / v) was 0.21 ± 0.02.

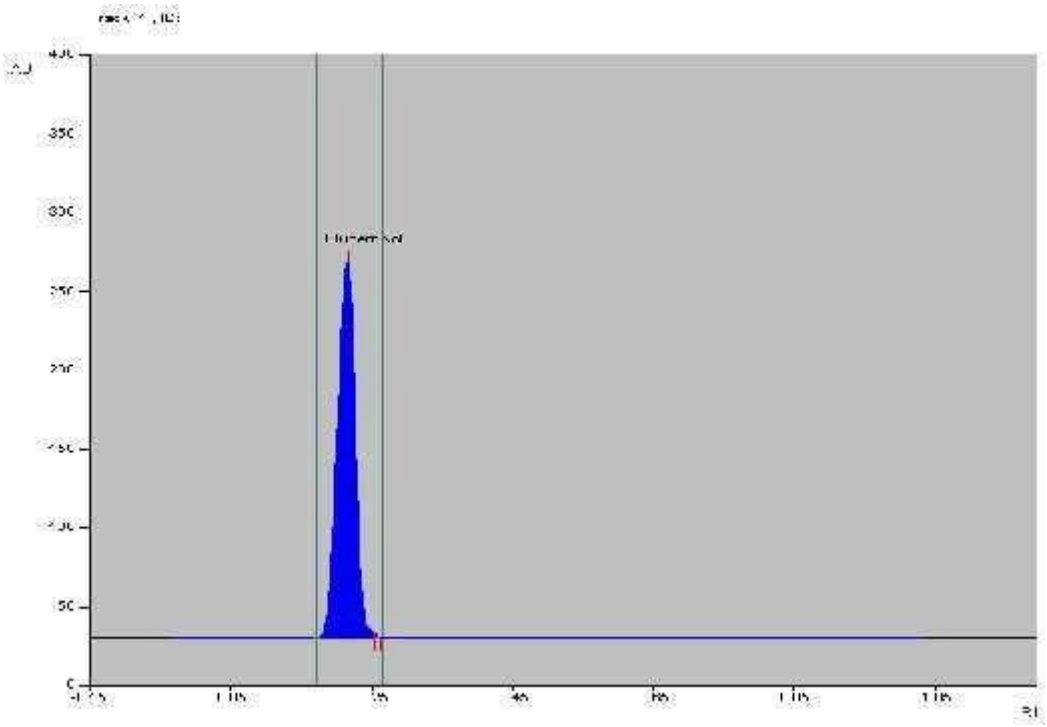


Fig: 5.17. Typical HPTLC Chromatogram of Flupentixol dihydrochloride at 229nm.

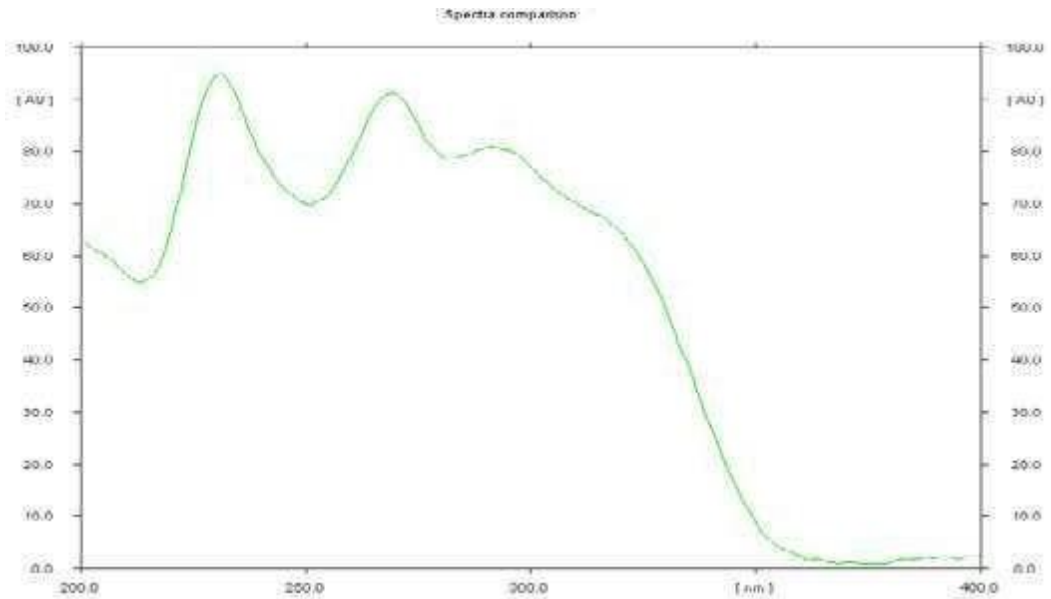


Fig: 5.18. Typical absorption spectrum obtained from standard Flupentixol dihydrochloride solution.

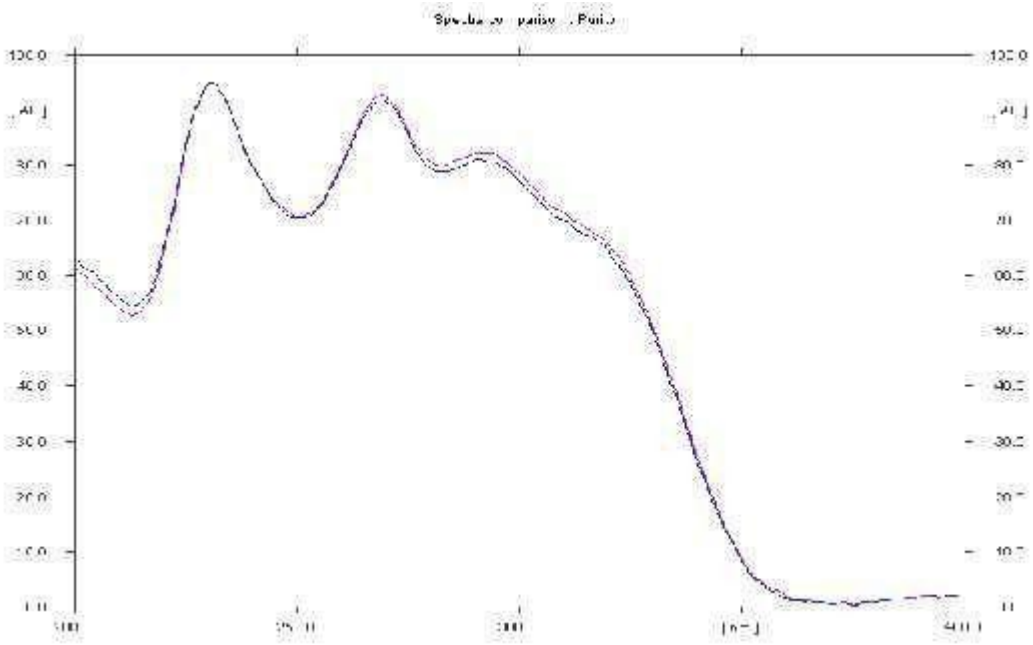


Fig 5.19: Overlay spectrum of sample and standard solution of Flupentixol dihydrochloride.

Table 5.31: Calibration data of Flupentixol dihydrochloride by HPTLC method.

SI. No.	Concentration (ng / spot)	R_F	Peak area
1	300	0.20	1286.4
2	600	0.21	2485.3
3	900	0.21	3607.1
4	1200	0.21	4668.2
5	1500	0.21	5732.8

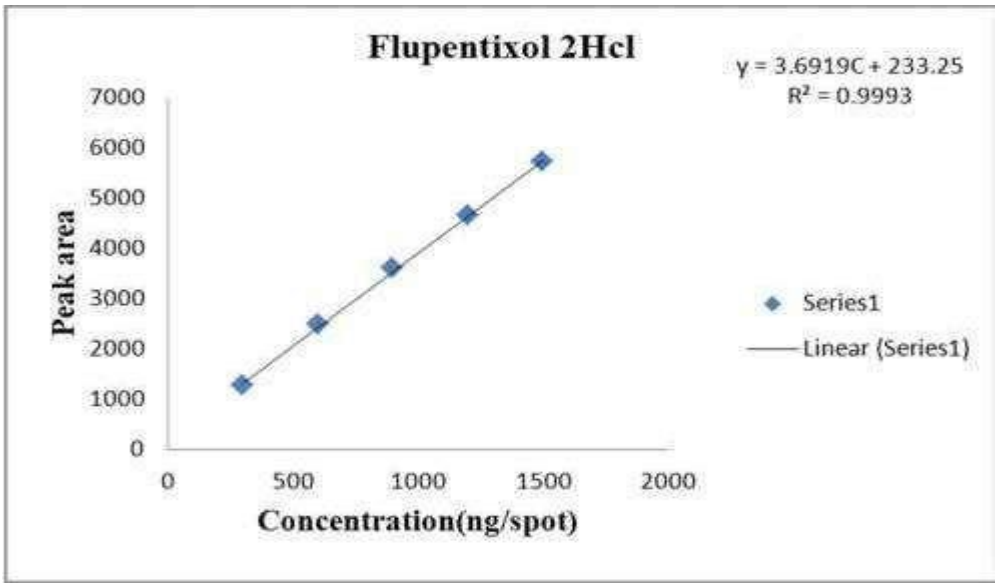


Fig: 5.20 Calibration curve of Flupentixol dihydrochloride by HPTLC method.

Table 5.32: Characteristic parameters for the proposed HPTLC method.

Parameters	HPTLC
Calibration range (ng / spot)	300-1500
Detection wavelength	229 nm
Mobile phase (Toluene : Glacial Acetic Acid)	7 : 3 (v / v)
R_F value	0.21± 0 .02
Regression equation (Y*)	Y = 3.6919C + 233.25
Slope (b)	3.6919
Intercept (a)	233.25
Correlation coefficient(r^2)	0.9993
Limit of detection (ng / spot)	6.24
Limit of quantitation (ng / spot)	18.92

*$Y = b\,C + a$, where C is the concentration of compound in ng / spot and Y is the peak area

1. LINEARITY

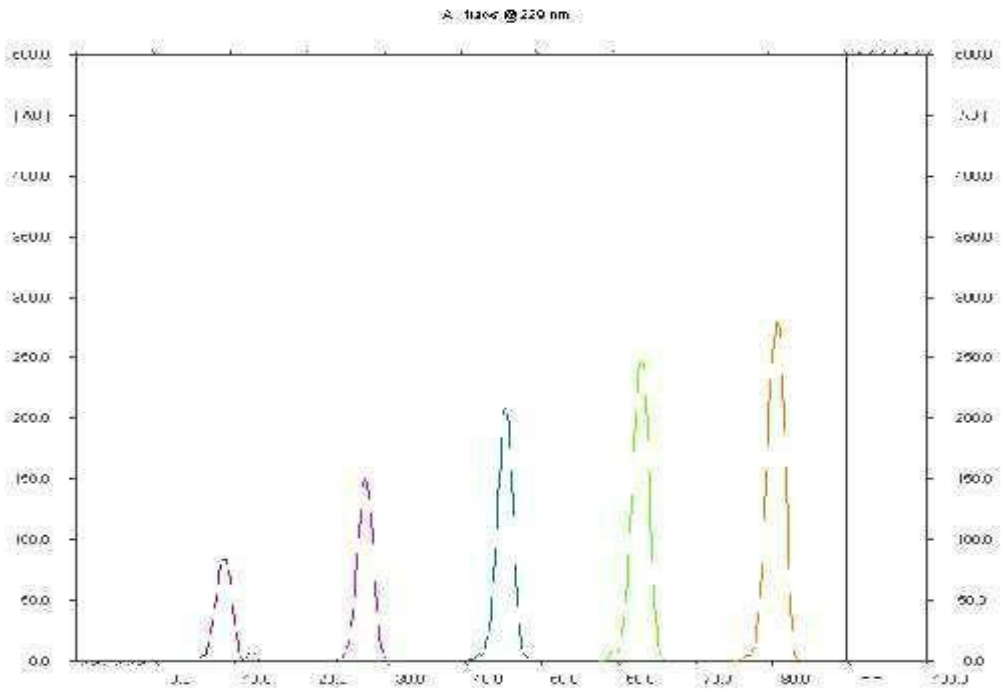


Fig 5.21: 3D of Linearity studies by HPTLC method.

2. PRECISION

The precision of the analytical method was studied by analysis of multiple sampling of homogeneous sample. The precision results were expressed as standard deviation Or relative standard deviation.

(a) System precision

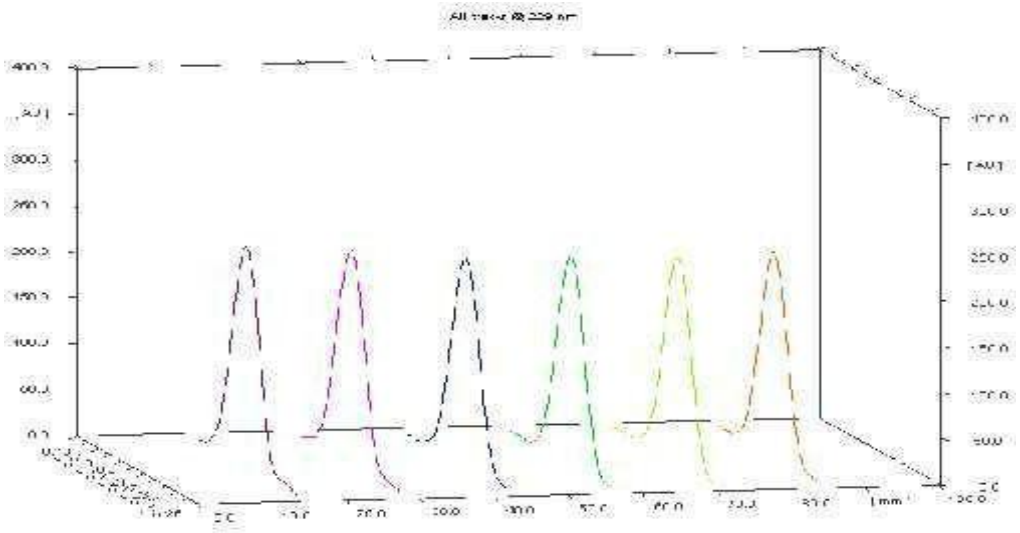


Fig: 5.22. 3D of System Precision by HPTLC method.

Table 5.33: System Precision of Flupentixol dihydrochloride by HPTLC method.

SI. No.	Concentration Peak area (ng / spot)	
1	900	3573.3
2	900	3610.6
3	900	3609.1
4	900	3527.6
5	900	3572.5
6	900	3562.6
Mean		3575.95
Std.Dev		31.09474
%RSD.		0.869552

(b) Method precision

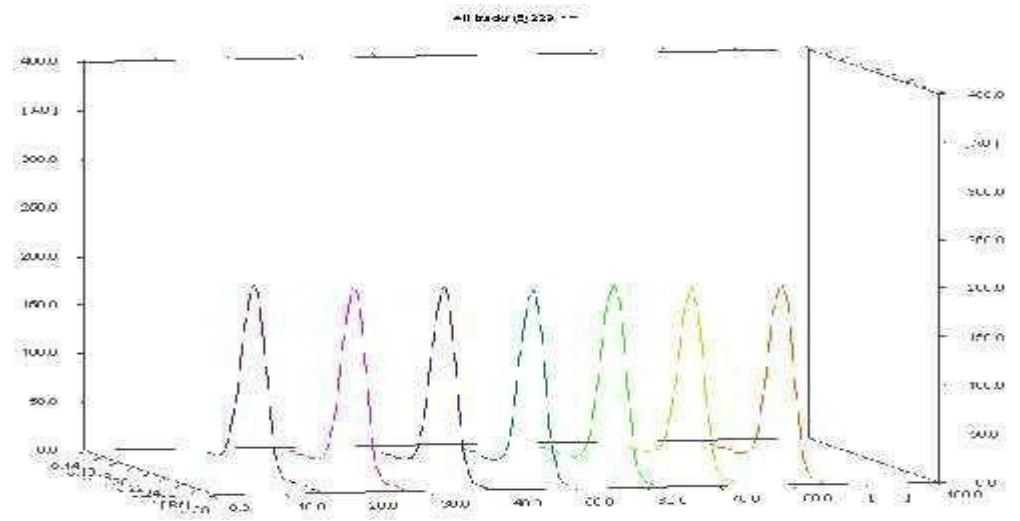


Fig: 5.23. 3D of Method Precision by HPTLC methodTable: 5.34.

Method Precision of Flupentixol dihydrochloride by HPTLC method

SI. No.	Concentration (ng / spot)	Peak area	% Assay
1	900	3498.6	98.29
2	900	3521.6	98.94
3	900	3501.5	98.37
4	900	3563.3	100.11
5	900	3551.1	99.77
6	900	3533.7	99.28
Mean		3528.3	99.12667
Std.Dev		26.15041	0.736442
%RSD.		0.741162	0.74293

3. ACCURACY

Table 5.35: Recovery studies of Flupentixol dihydrochloride by HPTLC method.

Tablet	Label claim (mg)	Spike Level (%)	Amount added ng / spot	Amount recovered ng / spot	Recovery ± SD (%)	% RSD
		50%	450	443.63	98.58 ± 0.416	0.422
Sample	3	100%	900	912.73	101.4 ± 0.414	0.409
		150%	1350	1343.63	99.52 ± 0.138	0.139

Average of three determinations.

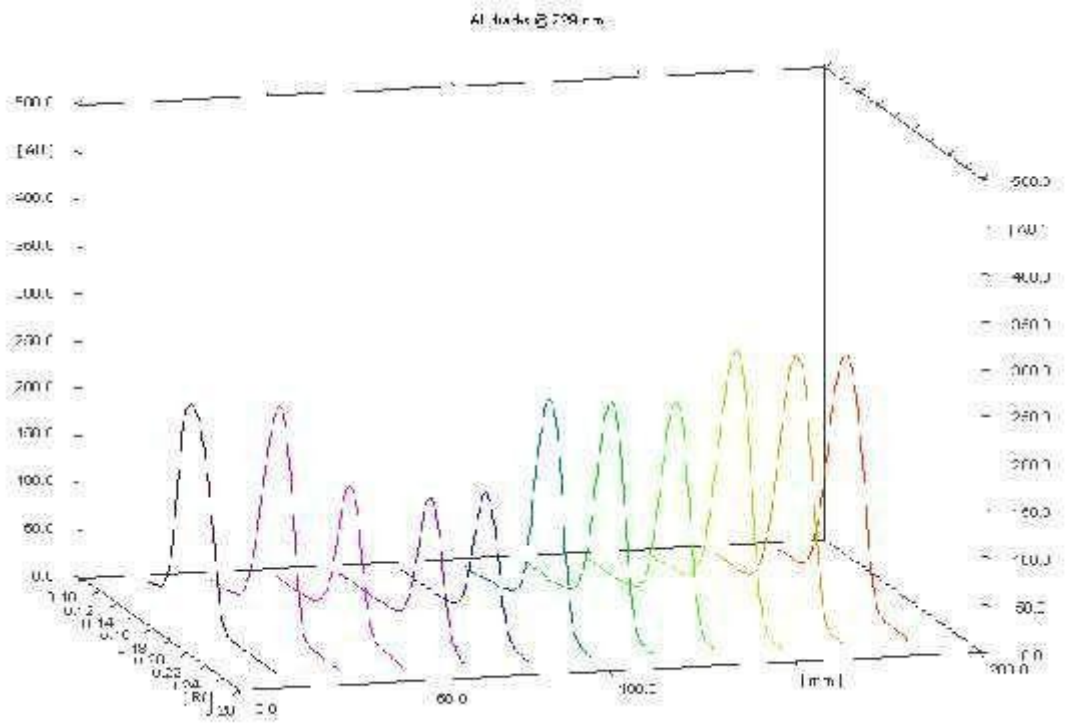


Fig 5.24: 3D of Accuracy of Flupentixol dihydrochloride by HPTLC method.

4. RUGGEDNESS

Table 5.36: Ruggedness studies of Flupentixol dihydrochloride by HPTLC method.

Tablet	Label claim (mg)	Analyst I		Analyst II	
		Amount found (mg)	Recovery ± SD (%)	Amount found (mg)	Recovery ± SD (%)
Sample	3	2.978	99.26 ± 0.10	3.003	100.1 ± 0.23

Mean ± S.D. from six determinations.

5. STABILITY

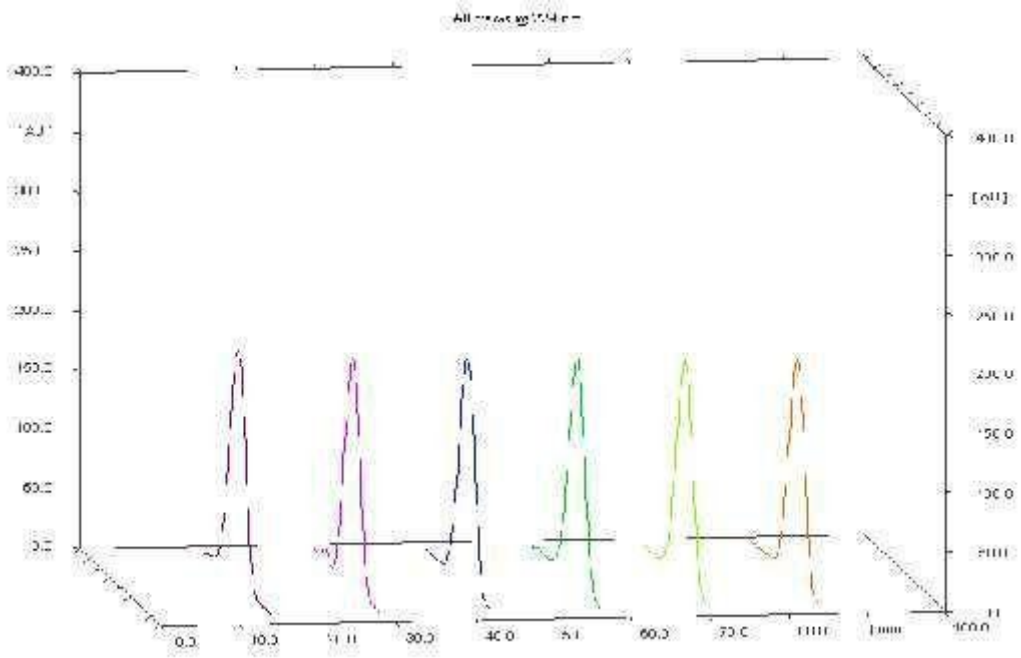


Fig 5.25: 3D of Stability studies by HPTLC method.

CHAPTER 6

DISCUSSION

UV Spectrophotometric, HPLC and HPTLC methods developed were found to be rapid, simple, precise, accurate and economic for routine estimation of Flupentixol dihydrochloride in bulk and pharmaceutical dosage forms.

PART A: UV SPECTROSCOPY

The spectrophotometric methods developed were.

Method A: Zero Order Derivative Spectroscopy Method B: First Order Derivative Spectroscopy Method C: Second Order Derivative Spectroscopy After considering the solubility and stability, Distilled water was selected as the common solvent. The absorption spectra were recorded in the wavelength region of 200-400 nm in UV method. The spectra

are presented as **Fig: 5.1, 5.3 and 5.5.**

Beer's law range was confirmed by the linearity of the calibration curve of Flupentixol dihydrochloride which were represented in **Fig: 5.2, 5.4, and 5.6.**

Flupentixol dihydrochloride showed linearity in the concentration range of 3-15 mcg /ml in Zero order, first order and second order derivative Spectroscopy, respectively.

The optical characteristics such as absorption maxima, Beer's law limits, molar absorptivity, slope (b), intercept (C), Sandell's sensitivity, correlation coefficient (r^2) obtained from different concentrations, percent relative standard deviation, LOD and LOQ values were presented in Table: 5.2, 5.7, and 5.12. The results showed that these methods have reasonable precision.

Results obtained with proposed methods confirm the suitability of these methods for pharmaceutical dosage forms. The accuracy of the methods were confirmed by the recovery studies, by adding known amount of the pure drug to the pharmaceutical formulation and the percentage recovery studies were determined and data were presented in analytical **Tables: 5.4, 5.9 and 5.14,** respectively. The results were within the range of 98.33 -100.87 % and were found to be highly accurate.

Ruggedness test expresses the precision of the method. The ruggedness results were shown in **Table: 5.5, 5.10 and 5.15,** respectively. The results were found to be highly precise.

The other active ingredients and common excipients present in the dosage forms of Flupentixol dihydrochloride did not interfere, when added in the mentioned concentration ranges to the drug and estimated by the proposed methods. The methods reported here are found to be simple, sensitive, accurate, precise, and economical can be used in the determination of Flupentixol dihydrochloride from pharmaceutical formulations in a routine manner.

PART B: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

In HPLC method, HPLC conditions were optimized to obtain, an adequate separation of eluted compounds. The objective of this study was to develop a rapid and sensitive RP-HPLC method for the analysis of Flupentixol dihydrochloride in bulk drug and pharmaceutical dosage form by using the most commonly employed RP C-18 column with UV-detection.

The run time was set at 6 min and the retention time for Flupentixol dihydrochloride was 4.28 ± 0.1 min. Each sample was injected 5 times and the retention times were same. When the concentrations of Flupentixol dihydrochloride and its respective peak areas were subjected to regression analysis by least squares method, a good linear relationship (r^2= 0.9996) was observed between the concentration of Flupentixol dihydrochloride and the respective peak areas in the range 10-50 mcg / ml. The regression equation was used to estimate the amount of Flupentixol dihydrochloride, either in tablet formulations or in validation study (precision and accuracy). For the proposed RP-HPLC method, characteristic parameters were shown in **Table: 5.16.**

To analyse tablet formulations, RP-HPLC method has been developed. Flupentixol dihydrochloride tablets (each containing 3 mg of the drug) were analyzed as per the procedure described above. The low % RSD values (≤ 2) indicated that the method was precise and accurate. The mean recoveries were found in the range of 98.95 –99.88 %. No interfering peaks were found in the chromatogram indicating that excipients used in the tablet formulation did not interfere with the estimation of the drug by the proposed RP-HPLC method.

The proposed RP-HPLC method was also validated for intra and inter-day variation. When the solution containing 30 mcg/ml of Flupentixol dihydrochloride was repeatedly injected on the same day, the %RSD in the peak area for six replicate injections was found to be 0.25%. Also the inter day variation (6 days and sixinjections) was found to be 1.62%. The results are presented in **Tables: 5.18.** The % RSD values were within 2 and the method was found to be precise.

Keeping the flow rate constant (1.0 ml / min), the chromatograms of drug solution were recorded by changing mobile phase ratios such as Methanol: Water = 60:40, 70:30 and 65:35(v/v). With the mobile phase Methanol: Water (65:35, v / v,), thepeaks were sharp with good resolution. The results are presented in **Table 5.28**. These values indicated that the method was quite robust.

Keeping the ratio of mobile phase constant Methanol: Water (65:35, v / v,), the chromatograms of drug solution were recorded with different flow rates such as 0.8 ml / min, 1.0 ml / min and 1.2 ml / min. With the flow rate of 1.0 ml / min, the peaks were sharp with

good resolution but with other flow rates results were not satisfactory. So 1.0 ml/min kept constant for the analysis. The results were presented in **Table: 5.25.**

The assay of Flupentixol dihydrochloride was performed by different analyst and on different dates (days). The % assay was calculated and those values were given in **Table: 5.24**. The results were reported to be within the limits.

PART C: HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

A full UV-Visible scan of Flupentixol dihydrochloride spots revealed two maxima at λ = 229 and 269 nm. The 229 nm was chosen for quantification. The R_F value of Flupentixol dihydrochloride after development with the mobile phase Toluene: Glacialacetic acid (7: 3, v/v) was 0.21 ± 0 .02. When the concentrations of Flupentixol dihydrochloride and its respective peak areas were subjected to regression analysis by least squares method, a good linear relationship (0.999) was observed between the concentration of Flupentixol dihydrochloride and the respective peak areas in the range 300-1500 ng/spot. The regression of Flupentixol dihydrochloride was found to be Y = 3.6919C + 233.25, where 'Y' is the peak area and 'C' is the concentration of Flupentixol dihydrochloride. The regression equation was used to estimate the amount of Flupentixol dihydrochloride, either in tablet formulations or in validation study (precision and accuracy). For the proposed RP-HPLC method, characteristic parameters were shown in **Table: 5.32.**

The precision of the method in terms of system precision (% RSD) was determined by analyzing Flupentixol dihydrochloride standard solution containing 900 ng / spot and method precision (% RSD) was assessed by analyzing the solution containing 900ng/spot. The results of the precision studies are shown in **Table: 5.33** and **5.34.**

Determination of method accuracy by the standard addition method at three concentration levels returned a mean recovery of 98.58 - 101.4 % and is given in**Table: 5.35.**

Ruggedness test expresses the precision of the method. The ruggedness results were Shown in **Table: 5.36.**

CHAPTER 7

CONCLUSION

Flupentixol dihydrochloride is chemically, (EZ)-2-[4-[3-[2-(trifluoromethyl) thioxanthen-9-

ylidene] propyl] piperazin-1-yl] ethanol.dihydrochloride. Development of methods to achieve the final goal of ensuring the quantity of drug substances and drug products is not a trivial undertaking. The capabilities of the four methods were complementary to each other.

Few analytical methods were appeared in the literature survey for the determination of Flupentixol dihydrochloride, which includes UV/Visible spectrophotometric methods, HPLC and HPTLC. In view of the above fact, some simple analytical methods were planned to develop with sensitivity, accuracy, precision and economical.

PART A: UV SPECTROSCOPY

In the present investigation, we have developed three simple and sensitive UV/Visible spectrophotometric methods for the quantitative estimation of Flupentixol dihydrochloride in bulk drug and pharmaceutical formulations.

The results of the developed methods are expressed in **Tables: 5.1** to **5.15.** In addition to positive requirements for analytical methods, the developed methods is that they are economical. advantage of all the presently.

The methods were validated in terms of linearity, accuracy, precision, ruggedness androbustness and used for the routine determination of Flupentixol dihydrochloride in Bulk drug and in pharmaceutical formulations.

PART B: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

In the present investigation, we have developed a simple, sensitive, precise and accurate RP-HPLC method for the quantitative estimation of Flupentixol dihydrochloride in bulk drug and pharmaceutical formulations. The results expressed in **Tables 5.16** to **5.30** for RP-HPLC method are promising. The RP-HPLC method is more sensitive, accurate and precise compared to the spectrophotometric methods.

PART C: HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

In HPTLC, the results of the developed method were expressed in **Tables: 5.31.** to **5.36.** In addition to positive requirements for analytical methods, the striking advantage of all the presently developed methods is that they are economical.

These methods can be used for the routine determination of Flupentixol dihydrochloride in bulk drug and in pharmaceutical formulation.

CHAPTER 8

SUMMARY

In the present work, an attempt was made to provide a newer, simple, accurate and low cost spectrophotometric and their derivative methods, one HPLC method and one HPTLC method for the effective quantitative determination of Flupentixol dihydrochloride as an active pharmaceutical ingredient as well as in pharmaceutical preparations, without the interferences of other constituent in the formulations.

For routine analytical purposes it is always of interest to establish methods capable of analyzing a large number of samples in a short time period with good accuracy and precision. The main purpose of this study was to develop accurate, precise and economic methods for the determination of Flupentixol dihydrochloride. Spectrophotometric technique, derivative method were applied without using any prior chemical pretreatment in the presence of the strongly overlapping spectra can generate large amounts of data within a short period of analysis. Pharmaceutical analysis occupies a pivotal role in statutory certification of drugs and their formulations either by the industry or by the regulatory authorities.

UV Spectrophotometry, RP-HPLC and HPTLC techniques have been used as tools in the present work. The above tools have been used for the development of new analytical methods for the assay of Flupentixol dihydrochloride. The contents of the thesis have been divided into ten chapters.

Chapter 1 opens with the introduction giving a brief account of analytical method development of drugs, followed by Part A which includes concepts of spectroscopy, general methodology of derivative methods. Part B includes brief account of HPLC method and information related to proposed method. Part C includes brief account of

HPTLC method. Part D is method validation which includes introduction, steps in validation, validation report and validation parameters for chromatographic methods. **Chapter 2** explains the objectives of present investigations adopted for the selected drug.

Chapter 3 for review of literature giving details on reported methods.

Chapter 4 consists of methodology which is divided into three parts, Part A, Part B and Part C. In Part A, the details about the proposed spectrophotometric methods on the drug are included. It gives information regarding the solubility of Flupentixol dihydrochloride which

was exploited in the present investigations for the development of three UV spectrophotometric methods which have been proposed for the determination of Flupentixol dihydrochloride respectively. In Part B, the details about the proposed RP-HPLC method and in Part C HPTLC method on the drug is included.

Method A is UV Spectrophotometric method which involves the determination of Flupentixol dihydrochloride in bulk drug and pharmaceutical formulations and has an absorption maximum at 229 nm in distilled water which is presented as **Fig: 5.1.** It obeys Beer's law in the concentration range of 3-15 mcg / ml.

Method B is First Order Derivative Spectrophotometric method which involves the determination of Flupentixol dihydrochloride in bulk drug and pharmaceutical formulations and has an absorption maximum at 222 nm in distilled water which is presented as **Fig. 5.3.** It obeys Beer's law in the concentration range of 3-15 mcg / ml.

Method C is Second Order Derivative Spectrophotometric method which involves the determination of Flupentixol dihydrochloride in bulk drug and pharmaceutical formulations and has an absorption maximum at 214 nm in distilled water which is presented as **Fig. 5.5.** It obeys Beer's law in the concentration range of 3-15 mcg / ml.

Part B is a RP-HPLC method in which determination of Flupentixol dihydrochloride was carried out on a RP-C_{18} column using a mobile phase consisting of Methanol.

Water (65:35 v / v). The mobile phase was pumped at a rate of 1.0 ml / min and the detection was carried out at 229 nm. The linearity was found to be in the range of 10- 50 mcg / ml and retention time was 4.28 ± 0 .1 min.

Part C is a HPTLC method in which determination of Flupentixol dihydrochloride wascarried out on a Pre coated silica gel 60 F_{254} on aluminium sheets using a mobile phase consisting of Toluene: Glacial acetic acid (7:3, v / v) and the detection was carried out at 229 nm. The linearity was found to be in the range of 300-1500 ng / spot and R_F was 0.21 ± 0 .02.

Chapter 5 consists of results which include, Part A, Part B and Part C respectively. InPart A, results obtained in each of the UV spectrophotometric method for estimation of Flupentixol dihydrochloride is summarized in **Tables: 5.1-5.15**. In Part B, the results obtained in RP-HPLC method for determination of Flupentixol dihydrochloride are summarized in **Tables:**

5.16-5.30 and in Part C, the results obtained in HPTLC method for determination of Flupentixol dihydrochloride aresummarized in **Tables: 5.31-5.36.**

Chapter 6 consists of discussion which discusses about the developed UV spectrophotometric, RP-HPLC and HPTLC methods.

Chapter 7 explains the conclusion in three parts, Part A, Part B and Part C respectively. Part A includes the proposed UV spectrophotometric methods for the quantitative estimation of Flupentixol dihydrochloride. Part B contains proposed RP- HPLC method for the quantitative estimation of Flupentixol dihydrochloride and Part C contains proposed HPTLC method for the quantitative estimation of Flupentixol dihydrochloride. These methods are validated in terms of sensitivity, accuracy and precision and can be used for the routine determination of Flupentixol dihydrochloridein bulk drug and pharmaceutical formulations.

Chapter 8 contains the summary.

Chapter 9 contains the bibliography.

CHAPTER 9
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