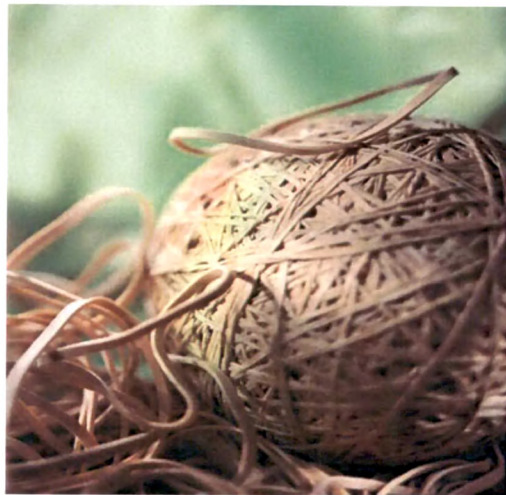


5. APPROACH TO THE DEVELOPMENT OF ANALYTICAL METHOD



5. APPROACH TO THE DEVELOPMENT OF ANALYTICAL METHODS.

5.1 SPECTROPHOTOMETRIC METHODS

The success of spectrophotometry in the field of analytical chemistry depends mainly on the easy manipulation and interpretation of absorption spectra. On the other hand, the traditional methods, based on the use of a restricted number of signals, proved to be often unsatisfactory for the analysis of multicomponent mixture, owing to their low accuracy and precision. Several techniques for the elaboration of spectrophotometric data have been proposed with the aim at extracting large analytical information from spectra composed of unresolved bands. Following this, some new analytical techniques have been applied for the analysis of single and multicomponent mixtures.¹

5.1.1 Difference spectrophotometry

The selectivity and accuracy of Spectrophotometric analysis of sample containing absorbing interference may be markedly improved by the techniques of difference spectrophotometry. The essential feature of a difference spectrophotometry is that the measured value, the difference in absorbance (A) between two equimolar solutions of the analyte in different chemical forms which exhibits different spectral characteristics. The criteria for applying difference spectrophotometry to the assay of a substance in the presence of other absorbing substance are that

- a. Reproducible changes may be induced in the spectrum of the analyte by the addition of the one or more reagent.
- b. The reagent does not affect the absorbance of the interfering substance.

The simplest and most commonly employed technique for altering the spectral properties of the analyte is the adjustment of pH by means of aqueous solution of acid, alkali or buffer.

5.1.2 Three wavelength method (Geometric correlation method)

This method is based on selecting 3 wavelengths (WL1, WL2, WL3). The three wavelengths selected to determine the concentration are based on the difference between the absorbance at the middle wavelength and a (base) line between the absorbance readings at the upper and lower wavelengths (figure 1.1). The order of the wavelength values affects the order in which data is acquired, but not the calculation. The intermediate wavelength among three assigned wavelengths is picked up as WL2

regardless of the order of the reading. In order to determine the concentration, the three wavelengths selected are as follows:

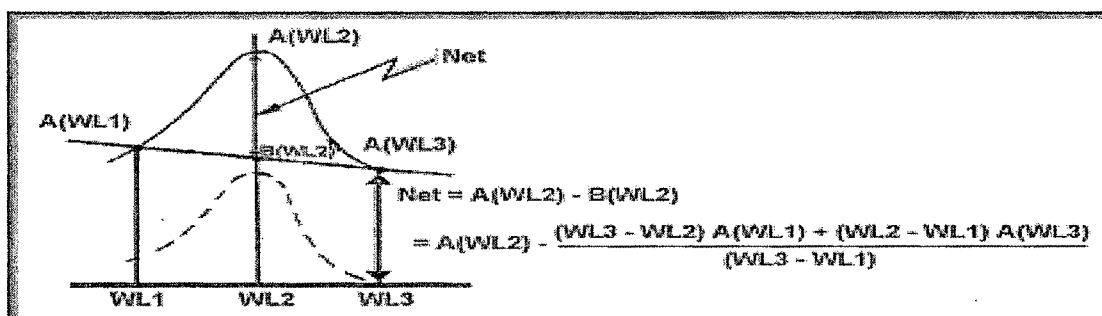
$$\text{WL1, WL2, WL3}$$

The absorbance is measured at these three wavelengths and the net absorbance is determined by using the following equation:

$$\begin{aligned} \text{Net} &= A(\text{WL2}) - B(\text{WL2}) \\ &= A(\text{WL2}) - \frac{(\text{WL3} - \text{WL2}) A(\text{WL1}) + (\text{WL2} - \text{WL1}) A(\text{WL3})}{(\text{WL3} - \text{WL1})} \end{aligned}$$

A graph of net absorbance vs. concentration of standard drug is plotted to calculate the regression equation.

Fig. 1.1: 3-wavelength photometric method



5.1.3 Derivative spectrophotometry

Derivative Spectrophotometry involves the conversion of a normal spectrum to its first, second or higher derivative spectrum.

The **first derivative (D^1)** spectrum 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 $dA/d\lambda$ vs. λ . The **second derivative (D^2)** spectrum is a plot of the curvature of the D^0 spectrum against wavelength or a plot of $d^2A/d\lambda^2$ vs. λ . In summary, the 1st 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. Two satellite maxima and an inverted band of which the minimum corresponds to the λ_{max} of the fundamental band characterize the 2nd derivative spectrum.

5.1.4 Absorption ratio (Q- analysis) spectrophotometry

It depends on the property that, for a substance, which obeys Beer's law at all wavelengths, the ratio of absorbance at any two wavelengths is a constant value independent of concentration or pathlength. Thus the method was developed for simultaneous estimation of EZE and statins in the same solution. In the quantitative assay of these drugs, absorbance are measured at two wavelengths-

- a. One being the λ_{max} of EZE (λ_1)
- b. Other being the wavelength of equal absorptivity (Isosbestic point).

5.1.5 First Derivative Zero Crossing spectrophotometry

The successive development of the zero-crossing technique (ZCT) was accordingly due to the feature of derivative spectra to show signals with either positive and negative value. This technique exploits the signal crossing through the abscissa axis, for a given component of a mixture, to assign the absorbance value to remaining components. ZCT resulted particularly effective in the analysis of several complex mixtures, when wide overlapping peaks were present in the corresponding zero-order spectrum.²⁻⁶ However, suitable analytical signals are often placed on the peak shoulders or characterized by a too low absorbance. This could heavily limit the accuracy and precision of the method, as the low stability of such signals is well known. The so-called "ratio spectra derivative" permits the use of the wavelengths corresponding to the maximum and also the use of the distance between consecutive maximum and minimum, thus avoiding these problems.⁷

5.1.6 Absorption Ratio First Derivative spectrophotometry

Salinas et al.⁸ developed a derivative quotient spectra method with a standard divisor when the spectra of the components are overlapped. The method was based on dividing the spectrum for a mixture by standard spectra for each of the analyses and designing the quotient to obtain a spectrum that is independent of the analyte concentration used as divisor. The use of standardized spectra as divisors minimize experimental errors background noise.⁹ an accurate choice of standard divisors and working wavelengths is fundamental for several reasons.^{10,11} Easy measurements on separate peaks, higher values of the analytical signals and no need to work only at zero-crossing points (sometimes co-existing compounds have no maximum or minimum at these wavelengths) are advantages for ratio spectra derivative spectrophotometry in comparison with the zero-crossing derivative spectrophotometry. Also, the presence of a lot of maxima and minima in ratio spectra

derivative data is another advantage, since these wavelengths give an opportunity for the determination of these compounds in the presence of other active compounds and excipients that possibly interfered with the assay.¹²

5.1.7 Difference Derivative Zero Crossing spectrophotometry

Derivative spectrophotometry offers greater selectivity than normal spectrophotometry in the simultaneous determination of two or more compounds without previous chemical separation. Derivative difference spectrophotometry¹³⁻¹⁵ will offer further advantages in canceling heavy spectral interferences during drug analysis when the irrelevant absorption is pH and solvent dependent. For difference measurements, alkaline solutions were placed in the reference cell and methanolic solutions in sample cell. The delta absorbance ($\Delta A = A_{\text{alk}} - A_{\text{acid}}$) ΔD_1 curves were recorded.

5.1.8 Quantitative Fourier Transform Infrared (FTIR) spectroscopy

Infrared (IR) spectrometry provides a useful tool for the identification of drugs.¹⁶⁻¹⁹ however, the traditional techniques employed to obtain the IR spectra, such as alkali halides disks, mulls and thin films, are not suitable for quantitative analysis. The Fourier transform (FT-IR) permits continuous monitoring of the spectral baseline for simultaneous analysis of different components of the same sample.^{20,21}

Quantitative analysis of the components in pharmaceutical preparations by FT-IR spectrometry is usually based upon the Lambert-Beer law. The principal problems are the ingredients presented in the pharmaceutical preparations. A common problem associated with all IR methods is that there are no specific or unique wavelengths of absorption for any of the components. The Fourier transform is somewhat superior to the filter option of spectrophotometers because the determination is not based on a single wavelength that is not unique for the analyte of interest.

5.1.9 Chemometric techniques²⁴⁻³²

Chemometric regression techniques can be classified into two-way regression techniques and multi-way regression techniques. Two way regression techniques include classical least squares (CLS), principal component regression (PCR), continuum regression and partial least squares with one (PLS-1) and two (PLS-2) dependent variable, while multi way regression techniques include N-way partial least squares (nPLS), parallel factor analysis (PARAFAC), trilinear decomposition (TLD) and multivariate curve resolution based on alternating least-squares (ALS).

5.1.9.1 Two way regression technique

Two way regression techniques use regression involving two variables. The main advantages of these techniques are the following: a higher speed of processing data concerning the values of concentrations and absorbance of compounds in the presence of the spectral interference, the errors of calibration model are minimized by measuring the absorbance at many points in the wavelength range of the zero order and derivative spectra. Chemometric calibration techniques in spectral analysis are gaining importance in the quality control of drugs in mixtures and pharmaceutical formulations containing two or more drugs with overlapping spectra due to no need of any separation procedure in the drug determinations. So, research in this area is important, aiming at the future acceptance of these methods by the regulatory agencies.

5.1.9.1.1 Classical least Square regression method

The CLS method is involved in the application of MLR to the classical expression of the Beer-Lambert law of spectrophotometry:

$$A = K \times C$$

This equation is a matrix equation and it can be written as a linear equation system:

$$\begin{aligned} A_1 &= K_{11}C_1 - K_{12}C_2 \dots \dots \dots K_{1c}C_c \\ A_2 &= K_{21}C_1 - K_{22}C_2 \dots \dots \dots K_{2c}C_c \\ &: \\ &: \\ A_w &= K_{c1}C_1 - K_{c2}C_2 \dots \dots \dots K_{wc}C_c \end{aligned} \quad (1)$$

where A_w is the absorbance at the w^{th} wavelength, K_{wc} is the calibration coefficient for the c^{th} component at the w^{th} wavelength, C_c is the concentration of the C^{th} component.

5.1.9.1.2 Inverse least square regression method

The ILS method uses the application of MLR to the Inverse expression of the Beer-Lambert law of spectrophotometry:

$$C = P \times A$$

This equation can be written as a linear equation system:

$$\begin{aligned} C_1 &= P_{11}A_1 - P_{12}A_2 \dots \dots \dots P_{1w}A_w \\ C_2 &= P_{21}A_1 - P_{22}A_2 \dots \dots \dots P_{2w}A_w \\ &: \\ C_c &= P_{c1}A_1 - P_{cw}A_w \dots \dots \dots P_{cw} \end{aligned} \quad (2)$$

Where, A_w is the absorbance at the w^{th} wavelength, P_{cw} is the calibration coefficient for the C^{th} component at the W^{th} wavelength; C_c is the concentration of the C^{th} component.

5.2 CHROMATOGRAPHIC METHODS

The modern methods of choice for quantitative analysis are HPLC, GLC and HPTLC, which are highly versatile and sophisticated. Chromatographic methods are commonly used in regulatory laboratories for the qualitative and quantitative analysis of drug substances, drug products, raw materials and biological samples throughout all phases of drug development, from research to quality control.

5.2.1 High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography²² (HPLC) is the fastest growing analytical technique for the analysis of drugs. Its simplicity, high specificity, and wide range of sensitivity make it ideal for the analysis of many drugs in both dosage forms and biological fluids. The rapid growth of HPLC has been facilitated by the development of reliable, moderately priced instrumentation and efficient columns.

5.2.2 High Performance Thin Layer Chromatography (HPTLC)

High Performance Thin Layer Chromatography²³ (HPTLC) is a classical separative technique that has enjoyed wide spread popularity particularly in the analysis of complex mixtures of natural origin. The technique is becoming a routine analytical tool due to its advantages of low operating cost, high sample throughput, and need for minimum sample cleanup. The major advantage of HPTLC is that several samples can be run simultaneously using a small quantity of mobile phase unlike HPLC, thus lowering analysis time and cost per analysis.

5.3 VALIDATION OF ANALYTICAL METHODS³³⁻³⁶

As defined by the USP, method validation provides an assurance of reliability during normal use, and is sometime referred to as "the process of providing documented evidence that the method does what it is intended to do." The objective of validation of an analytical method is to demonstrate that the procedure, when correctly applied, produces results that are fit for purpose. To be fit for the intended purpose, the method must meet certain validation characteristics.

5.3.1 Spectrophotometry

Typical validation characteristics, which should be considered, are: selectivity (specificity), linearity, range, accuracy, precision, limit of detection, limit of quantitation, ruggedness and robustness.

5.3.1.1 Selectivity (Specificity): Selectivity of a method refers to the extent to which it can determine particular analyte(s) in a complex mixture without interference from other components in the mixture. The terms selectivity and specificity have often been used interchangeably. The term specific generally refers to a method that produces a response for a single analyte only, while the term selective refers to a method that provides responses for a number of chemical entities that may or may not be distinguished from each other.

Degradation information obtained from stress studies (e.g., products of acid and base hydrolysis, thermal degradation, photolysis and oxidation) for the drug substance and for the active ingredient in the drug product should be provided to demonstrate the specificity of the assay and analytical procedures for impurities. The stress studies should demonstrate that impurities and degradants from the active ingredient and drug product excipients do not interfere with the quantitation of the active ingredient. Stress studies are described in various FDA guidance relating to the stability of drug products.

5.3.1.2 Linearity: The linearity is the ability of analytical procedure to produce test results which are proportional to the concentration (amount) of analyte in samples within a given concentration range, either directly or by means of a well-defined mathematical transformation. Linearity should be determined by using a minimum of six standards whose concentration span 80 -120% of the expected concentration range. The linearity of a method should be established by visual inspection of a plot of analytical response as a function of analyte concentration. If there is a linear relationship, test results should be evaluated by appropriate statistical methods, for example, by calculation of the regression line by the method of least squares. In some cases, the test data may need to be subjected to a mathematical transformation prior to regression analysis. Reports submitted must include the slope of the line, intercept and correlation coefficient data. The measured slope should demonstrate a clear correlation between response and analyte concentrations. The results should not show

a significant deviation from linearity, which is taken to mean that the correlation coefficient, $r > 0.99$, over the working range (50-120 %).

5.3.1.3 Range : The specified range is normally derived from the linearity studies. The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample for which it has been demonstrated that the analytical method has suitable levels of precision, accuracy and linearity.

5.3.1.4 Accuracy: The accuracy of an analytical method is defined as the degree to which the determined value of analyte in a sample corresponds to the true value. Accuracy may be measured in different ways and the method should be appropriate to the matrix. The accuracy of an analytical method may be determined by:

Standard addition method: In the standard addition method, a sample is assayed, a known amount of pure active constituent is added, and the sample is again assayed. The difference between the results of the two assays is compared with the expected answer.

In both methods (spiked-placebo recovery and standard addition method), recovery is defined as the ratio of the observed result to the expected result expressed as a percentage.

% Recovery calculated by formula;

$$\% \text{ Recovery} = \frac{N(\sum xy) - (\sum x)(\sum y)}{N(\sum x^2) - (\sum x)^2} \times 100$$

N = Number of observations

Y = Amount of drug found

X = Amount of standard drug- added

5.3.1.5 Precision: The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained *from* multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

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

(b) **Reproducibility:** Reproducibility expresses the precision between laboratories. For these guidelines, a simple assessment of repeatability will be acceptable. The precision of an analytical procedure is usually expressed as the variance, standard

deviation or coefficient of variation of a series of measurements. A minimum of 5 replicate sample determinations should be made together with a simple statistical assessment of the results, including the percent relative standard deviation.

5.3.1.6 Limit of Detection (LOD): The lowest amount of an analyte in a sample that can be detected, but not necessarily quantified as an exact value is termed as limit of detection. The LOD may be determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level (lowest calibration standard) at which the analyte can be reliably detected. The lowest calibration standard which produces a peak response corresponding to the analyte should be measured *n* times (normally 6-10). The standard deviation of response of analytical blank (*X*) and the slope of calibration curve (*S*) calculated by formula;

$$\text{LOD} = 3.3 \times (X / S).$$

5.3.1.7 Limit of Quantitation (LOQ): The lowest amount of the analyte in the sample that can be quantitatively determined with defined precision under the stated experimental conditions is termed as limit of quantitation. The LOQ may be determined by preparing standard solutions at, estimated LOQ concentration. The solution should be injected and analyzed '*n*' times. The average response and the standard deviation should be calculated and the SD should be less than 2.0 %. If the SD exceeds beyond the critical value, a new standard solution of higher concentration should be prepared and the above procedure repeated. It can be calculated by formula;

$$\text{LOQ} = 10 \times (X / S).$$

5.3.1.8 Ruggedness: Ruggedness 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, different instruments, different lots of reagents, different elapsed assay times, different assay temperatures, different days etc.

5.3.1.9 Robustness: It is the measure of capability of analytical method to remain unaffected by small but deliberate variation in the method parameters and provides an indication of its reliability during normal range.

5.3.2 Chemometric methods

Using the cross-validation method, the following statistical parameters have been obtained:

Root mean square error of prediction (RMSEP) (model diagnostic):

The RMSEP is a diagnostic parameter for examining the errors in the predicted concentrations. While the statistical prediction error quantifies precision, RMSEP summarizes both precision and accuracy. RMSEP is calculated from the following equation

$$\text{RMSEP} = \sqrt{\frac{\sum (C_i - C)^2}{n}}$$

Where, C_i is the true concentration of the component of interest in the i^{th} sample, C is the predicted concentration and n is the number of samples.

The RMSEP summarizes the spread or the concentration errors into one number similar to a standard deviation and in the same units as the concentration values. The full range of concentration residuals should correspond to approximately 2-3 RMSEP units if there is no bias

The predicted versus known concentration plot (model and sample diagnostic)

The predicted concentrations of the validation samples were plotted against the known concentration values. This tool is used to determine whether the model accounts for the concentration variation in the validation set or not. Plots were expected to fall on a straight line with a slope of 1 and 0 intercept.

Concentration residuals versus actual concentration plot (model and sample diagnostic)

The difference between the known and the predicted concentration (residuals) were plotted against the actual concentrations for the validation samples. This tool is used to determine whether the model accounts for the concentration variation in the validation set and it also provides information about how well the method will predict future samples.

5.3.2.1 Calibration set (Training set)

Calibration set design can be of central composite design, factorial design or orthogonal set and non orthogonal set. Calibration range should be such that it should include dose ratio in commercial formulation and at the same time the absorbance in wavelength region selected should not be outside linearity limit.

5.3.3 Chromatography

Typical validation characteristics, which should be considered, are: selectivity (specificity), linearity, range, accuracy, precision, limit of detection, limit of

quantitation, ruggedness, robustness and system suitability testing that is additional parameter.

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

Number of theoretical plates (N) is a measure of column efficiency. It is calculated by formula

$$N = 16 (T_R/W)^2 \text{ or } N = 5.54 (T_R/W_{1/2})$$

T_R = Retention time of the substance.

W = Width of peak measured by extrapolating the relatively straight sides to the base line for respective substances

$W_{1/2}$ = Peak width at half height.

It is a measure of band spreading of a peak. Smaller the band spread higher is the number of theoretical plates indicating good system performance. Columns with N ranging from 5,000 to 1,00,000 plates/meter are ideal for a good system.

Resolution (R) is a function of column efficiency and is specified to ensure that closely eluting compounds are resolved from each other, to establish the general resolving power of the system, and to ensure that internal standards are resolved from the drug. For baseline separation, the ideal value of R , is 1.5. It can be calculated by formula;

$$R = 2 (T_{R2} - T_{R1}) / W_1 + W_2$$

T_{R1} and T_{R2} = Retention of respective substances

W_1 and W_2 = Width of peak measured by extrapolating the relatively straight sides to the base line for respective substances

Relative standard deviation (RSD) is calculated from data of five replicate injections of analyte, if the requirement is less than 2.0 % or less; data from six replicate injections are used if RSD requirement is more than 2.0 %.

The **tailing factor (T)**, measure of peak symmetry, is unity for perfectly symmetrical peaks and its value increase as tailing becomes more pronounced. In some cases, value less than unity may be observed. As peak asymmetry increases, integration, and hence precision, becomes less reliable. Tailing factor calculated by formula;

$$T = W_{0.05} / 2F$$

$W_{0.05}$ = Width of peak at 5 % height ,

F = distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at 5 % height

Asymmetry factor (As) can be used as a criterion of column performance. The peak half width, b , of a peak at 10% of the peak height, divided by the corresponding front half width, a , gives the asymmetry factor.

$$As = b/a$$

For a well packed column, an asymmetry factor of 0.9 to 1.1 should be achievable.

The **selectivity (or separation factor – α)**, is a measure of relative retention of two components in a mixture. The ideal value of α is 2. it can be calculated by using the formula,

$$\alpha = (V_2 - V_0) / (V_1 - V_0)$$

where, V_0 is the void volume of the column or solvent front.

V_2 and V_1 are the retention volumes or times of the first and the second peak, respectively.

5.4 BIOANALYTICAL METHOD (Human plasma)

Study of drug metabolites it is necessary to have highly sensitive analytical methods. Methods which can analyze drug in present or absence of its metabolites in biological fluid is term as bioanalytical methods. HPLC provides highly selective bioanalytical methods, but sample preparation is very important in bioanalytical method.

5.4.1 Sample preparation: Achieving good quality result can be translated in to simple term:

Cleaner Sample = Better Results

Sample preparation: Speed, size, ease of use, cost, and reliability, ruggedness

Instrumentation: Selectivity, Resolution, Sensitivity, LOD, Precision, Accuracy.

Sample can be propagated for HPLC analysis in wide variety of forms either directly or after some treatment. The sample preparation in Bio-equivalence or bioavailability study of drug involved following steps.

Step 1. Separation of plasma from blood sample: Blood sample is taken from subjected body in heparinised tube and centrifuged at 3000rpm for 15 minutes at 10°C. The plasma, was separated from the blood tissues by pipetting out the supernatant layer of plasma.

Step 2. Storage of plasma: The plasma samples were stored in deep freezer at -70°C until the time of analysis.

Step 3. Thawing and addition of internal standard to sample: The frozen plasma samples were allowed to thaw at room temperature and then vortexed to ensure complete mixing of content.

Step 4. Extraction procedure: Three types of extraction procedure are used in bioequivalence study--:

1). Classical Techniques

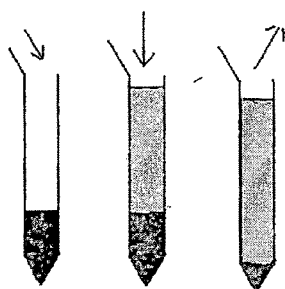
A) Protein precipitation.

B) Liquid-liquid extraction.

2). New Technique

C) Solid phase extraction.

A). Protein precipitation method: Highly water soluble compounds are extracted by protein precipitation method. Commonly used precipitating agents are acetonitrile, methanol, perchloric acid etc. It is probably the most popular and usually the first choice for sample preparation used in the pharmaceuticals industry. This method is suitable for polar compounds.



Steps: -

Pipette 200µL of plasma

Pipette 400-600µL acetonitrile.

Vortex.

Collect supernatant

Dilute with solvent and inject.

Protein Precipitation is fast method to produce analyzable sample, however in term of cleanliness, the sample is considered crude. The technique is also referred as the quick and dirty way.

Most Solvent used: -

(1) organic precipitating agent: -

Methanol

Acetonitrile

Ethanol

(2) Inorganic precipitating agent: -

Perchloric acid

Zinc sulphate

B). Liquid –liquid extraction.

In this process, process components of interest are partitioned between two immiscible Liquids, one being organic and other aqueous liquid phase to extract them. The aqueous phase can be adjusted to neutral, acidic or basic pH to get optimum extraction of drug.

This method is mainly used when compound is non-polar in nature.

Organic phase are :

Dichloro methane

Diethyl ether

Ethyl acetate

Tertiary butyl methyl ether

N-hexane

Aqueous phase are:

Neutral condition

Acidic condition

Basic condition

“It also define as the process of separating one constituent from a mixture by dissolving it into a solvent in which it is soluble but in which the other constituent of mixture are not”

Steps-:

Pipette 1mL of diluted plasma

Pipette 1mL of organic solvent (i.e. ethyl acetate)

Shake for 5 minutes

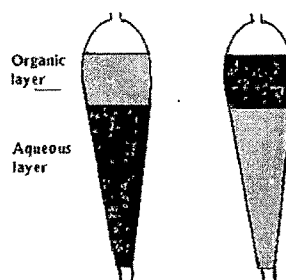
Remove organic layer (freeze if aqueous is on the bottom)

Dry organic layer (i.e. magnesium sulfate)

Evaporate organic layer to dryness

Reconstitute in mobile phase

Overall liquid-liquid extraction offers a better clean up than protein precipitation ,by using added variants such as back washing, back extraction, evaporation and drying. The technique, on the other hand is extremely time consuming.

**C). Solid phase extraction-:**

“ The solid phase extraction process consist of putting a solution containing the analyte dissolve in a suitable solvent on a column filled with a solid sorbent. If SPE has been successful, the analyte of interest will have been completely absorbed onto the solid phase to be subsequently desorbed by an appropriate solvent”

Materials can be used for a SPE sorbent:-

The choice of the packing material depend on the nature of the analyte and the matrix.

Reverse Phase : C18,C8,C4,Butyle ,phenyl, cyclohexyl.

Normal Phase : Silica, Diol, Aminopropyle,Cyanopropyle.

Ion Exchange : Strong or weak cation /Anion Exchanger.

Mostly prefer cartridges are HLB(hydrophilic lipophilic balance)

Hydrophilic monomer - N-vinyl pyrrolidone

Lipophilic monomer - Di vinyl benzene

Advantages of SPE extraction:

Provides high &reproducible recoveries for acidic, basic and neutral analytes

easy to use and rugged, easy to automate for high sample throughput.

5.4.2 Bioanalytical method validation³⁵

Selective and sensitive analytical methods for the quantitative evaluation of drugs and their metabolites (analytes) are critical for the successful conduct of preclinical and/or biopharmaceutics and clinical pharmacology studies.

A. Full Validation: Full validation is important when developing and implementing a bioanalytical method for the first time.

B. Partial Validation: Partial validations are modifications of already validated bioanalytical methods.

C. Cross-Validation: Cross-validation is a comparison of validation parameters when two or more bioanalytical methods are used to generate data within the same study or across different studies.

Typical method development and establishment for a bioanalytical method include determination of (1) selectivity, (2) accuracy, precision, recovery, (3) calibration curve, and (4) stability of analyte in spiked samples.

(1) Selectivity: Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. For selectivity, analyses of blank samples of the appropriate biological matrix (plasma, urine, or other matrix) should be obtained from at least six sources. Each blank sample should be tested for interference, and selectivity should be ensured at the lower limit of quantification (LLOQ).

(2) Accuracy, Precision, and Recovery: The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. Accuracy is determined by replicate analysis of

samples containing known amounts of the analyte. The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. Precision is further subdivided into within-run, intra-batch precision or repeatability, which assesses precision during a single analytical run, and between-run, inter-batch precision or repeatability, which measures precision with time, and may involve different analysts, equipment, reagents, and laboratories.

The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. Recovery pertains to the extraction efficiency of an analytical method within the limits of variability. Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise, and reproducible. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with unextracted standards that represent 100% recovery.

(3) Calibration/Standard Curve: A calibration (standard) curve is the relationship between instrument response and known concentrations of the analyte. A calibration curve should be prepared in the same biological matrix as the samples in the intended study by spiking the matrix with known concentrations of the analyte. A calibration curve should consist of a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard), and six to eight non-zero samples covering the expected range, including LLOQ.

(4) Stability: Drug stability in a biological fluid is a function of the storage conditions, the chemical properties of the drug, the matrix, and the container system. The stability of an analyte in a particular matrix and container system is relevant only to that matrix and container system and cannot be extrapolated to other matrices and container systems. Stability procedures evaluate the stability of the analytes during sample collection and handling, after long-term (frozen at the intended storage temperature) and short-term (bench top, room temperature) storage, and after going through freeze and thaw cycles and the analytical process. Conditions used in stability experiments reflect situations likely to be encountered during actual sample handling and analysis. The procedure should also include an evaluation of analyte stability in stock solution.

All stability determinations were made using a set of samples prepared from a freshly made stock solution of the analyte in the appropriate analyte-free, interference-free biological matrix. Stock solutions of the analyte for stability evaluation were prepared in ACN at known concentrations.

1. Freeze and Thaw Stability

Analyte stability was determined after three freeze and thaw cycles. Three aliquots at each of the low and high concentrations were stored at the intended storage temperature for 24 hours and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 12 to 24 hours under the same conditions. The freeze-thaw cycle was repeated two more times, then analyzed on the third cycle.

2. Short-Term Temperature Stability

Three aliquots of each of the low and high concentrations were thawed at room temperature and kept at this temperature from 4 to 24 hours (based on the expected duration that samples will be maintained at room temperature in the intended study) and analyzed.

3. Long-Term Stability

The storage time in a long-term stability evaluation should exceed the time between the date of first sample collection and the date of last sample analysis. Long-term stability was determined by storing at least three aliquots of each of the low and high concentrations under the same conditions as the study samples. The volume of samples was sufficient for analysis on three separate occasions. The concentrations of all the stability samples were compared to the mean of back-calculated values for the standards at the appropriate concentrations from the first day of long-term stability testing.

4. Stock Solution Stability

The stability of stock solutions of drug and the internal standard was evaluated at room temperature for at least 6 hours. It was also checked for refrigerated solution. After completion of the desired storage time, the stability should be tested by comparing the instrument response with that of freshly prepared solutions.

(VII) Matrix effect

The *recovery* of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic

standard. Recovery pertains to the extraction efficiency of an analytical method within the limits of variability. Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise, and reproducible. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with unextracted standards that represent 100% recovery.

5.5 STABILITY INDICATING ANALYTICAL METHOD³⁷

The parent drug stability test guideline Q1A (R2) issued by International Conference on Harmonization (ICH)³⁷ suggests that stress studies should be carried out on a drug to establish its inherent stability characteristics, leading to identification of degradation products and hence supporting the suitability of the proposed analytical procedures. It also requires that analytical test procedures for stability samples should be stability indicating and they should be fully validated. Accordingly, the aims of the present study were to establish inherent stability of EZE, SIMVA, LOVA, ROSUVA, PRAVA and also EZE combination with statins through stress studies under a variety of ICH recommended test conditions^{35,37}, and to develop a stability indicating assay³.

Stability Indicating Method (SIAM)

Method employed for analysis of stability samples in pharmaceutical industry with advent of ICH guidelines, requirement of SIAM has become mandatory. Guidelines require conduct of forced decomposition study (stress testing) under a variety of conditions like pH, light, oxidation, dry heat etc & separation of drug from degradation products. Methods reported as SIAM in last 3-4 decades fall short of current requirements

Regulatory status of SIAM

ICH guidelines – Law in EU, Japan & US & used by other countries

Q1A:- stability testing of New Drug Substances & Products

Q3B:- Impurities in New Drug products

Q6A:- Gives requirement of SIAMs under Universal Criteria for drug substances & drug products

Definition of SIAM as per USFDA Stability guidelines -1998

Validated quantitative analytical methods that can detect the changes with time in chemical, physical or microbiological property of drug substance & drug products &

are specific so that contents of active ingredient, degradation products & components of interest can be accurately measured without interference.

Other regulatory guidelines

WHO Guidelines for stability testing of pharmaceutical products containing well established drug substances in conventional dosage forms (Technical report 863, 1996)

CPMP- Note for guidance on stability testing of Active substances & related finished products –Committee for proprietary Medicinal products EMIA London 1998.

TDP- Stability testing of existing drug substances & products therapeutic products Directorate –Ottawa-Canada 1997

USP-27- Under stability studies on manufacturing says that samples of products should be assayed for potency by SIAM. Requirement is not explicit in other pharmacopoeias

Development of Validated SIAM

Based on HPLC method which accounts for 85-90 % of methods reported in literature.

STEP 1. Critical study on drug structure to assess the likely decomposition products

STEP 2. Collection of information on physicochemical property

STEP 3. Stress (forced decomposition) study

STEP 4. Preliminary separation study on stress samples

STEP 5. Final method development and optimization

STEP 6. Identification and characterization of degradation products and preparation of standards

STEP 7. Validation of SIAM

Accuracy: By spiking drug to placebo/formulation and determining percent recovery.

Better approach is to spike drug to degraded solution

Linearity: 0-100 % as drug may fall to very low concentration, may be narrowed as required, e.g. 80-120 % for bulk drugs and stable formulations and 50-120 % for injections and degradable entity. For degradation products 0-20 %

LOD & LOQ – Not important for actives but required for degradation products

Robustness – Similar to conventional methods

Trauma of Establishing SIAM for Combination Products

Effort is compounded due to separation of multiple drugs from one another and then from degradation products. Virtually impossible for formulation containing 6-10 drugs and subjected to forced degradation. Best option is to target separation of degradation product formed only in long term/ accelerated testing or limited stress testing.

Stress Testing

Stress testing of the drug substance can help identify the likely degradation products, which can in turn help establish the degradation pathways and the intrinsic stability of the molecule and validate the stability indicating power of the analytical procedures used. The nature of the stress testing will depend on the individual drug substance and the type of drug product involved.

Stress testing is likely to be carried out on a single batch of the drug substance.

It should include the effect of temperatures (in 10°C increments (e.g., 50°C, 60°C, etc.) above that for accelerated testing), humidity (e.g., 75% RH or greater)

Where appropriate, oxidation, and photolysis on the drug substance. The testing should also evaluate the susceptibility of the drug substance to hydrolysis across a wide range of pH values when in solution or suspension.

Photostability testing should be an integral part of stress testing. The standard conditions for photostability testing are described in ICH Q1B.

Examining degradation products under stress conditions is useful in establishing degradation pathways and developing and validating suitable analytical procedures.

However, it may not be necessary to examine specifically for certain degradation products if it has been demonstrated that they are not formed under accelerated or long term storage conditions.

Results from these studies will form an integral part of the information provided to regulatory authorities.

Q1B: Photostability Testing of New Drug Substances and Products

The tripartite harmonised ICH guideline was finalised (Step 4) in November 1996. This forms an annex to the main stability guideline, and give guidance on the basic testing protocol required to evaluate the light sensitivity and stability of new drugs and products.

Stability studies are necessary to strengthen the quality management for a global acceptance.

5.7 Statistical methods used to study and compare analytical methods.

5.7.1 Analysis of Variance (ANOVA)

One way ANOVA is used to create a null hypothesis that there was no difference between the means of the replicates and the alternate hypothesis that at least one sample mean was different. In order to apply this technique to m replicates of n samples of the same thing, calculate the sum of squares among the samples (SS_A) within the replicates (SS_w), and for the total data set (SS_T).

$$SS_T = \sum_j^m \sum_i^n x_{ij}^2 - \frac{(\sum_j^m \sum_i^n x_{ij})^2}{N}$$

$$SS_A = \sum_j^m \left[\frac{(\sum_i^n x_{ij})^2}{n} \right] - \frac{(\sum_j^m \sum_i^n x_{ij})^2}{N}$$

$$SS_w = SS_T - SS_A$$

Where, $N = n \times m$ Then arrange these results into a table that has the following entries.

Table 1.1: Result of one way ANOVA

Source	Sum of Squares	Deg. of Freedom	Mean Squares	F-test
Among Samples	SS_A	$M - 1$	$MS_A = \frac{SS_A}{m - 1}$	$F = \frac{MS_A}{MS_w}$
Within Replicates	SS_w	$N - m$	$MS_A = \frac{SS_A}{N - m}$	
Total	SS_T	$N - 1$		

Two way ANOVA: For data set containing n samples and m different treatments of that data.. In this case the ANOVA looks at the variance between samples by going down the columns as above, but also at the variance between (treatments by going across the columns of the data matrix. The table created for a two way ANOVA where the first F-test tests the significance of differences between samples and the second F-test tests the significance between treatments. In this twoway NOV A there are two additional sum of squares to compute, the sum of squares among treatments (SS_B) and the sum of squares of errors (SS_e). They are given by:

$$SS_A = \sum_i^n \frac{(\sum_j^m x_{ij})^2}{m} - \frac{(\sum_j^m \sum_i^n x_{ij})^2}{N}$$

$$SS_e = SS_T - (SS_A + SS_B)$$

Table 1.2: Result of two way ANOVA

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F-Tests
Among Samples	SS_A	$m - 1$	MS_A	$\frac{MS_A}{MSe}$
Among Treatments	SS_B	$N - 1$	MS_B	$\frac{MS_B}{MSe}$
Error	SS_e	$(m - 1)(n - 1)$	MSe	
Total Variation	SS_T	$N - 1$		

5.7.2 F test

Used for precision study and in ANOV A.

Description:

The hypothesis test is:

$$H_0: s_1 = S_2$$

$$H_a: s \neq S_2$$

$$\text{Test Statistic: } F = S_{12}/S_{22}$$

where S_1 and S_2 are the sample standard deviations

Significance level: Typically set to 0.05

Critical Region: $F < f(1-\alpha/2)(v_1, v_2)$ and, $F > f(\alpha/2)(v_1, v_2)$

where the critical region is determined from the F distribution function with $(N_1 - 1)$ and $(N_2 - 1)$ degrees of freedom and a significance level of 0.05.

Conclusion: Reject null hypothesis if T in critical region

5.7.4 T test

T Test procedure compares the means of two groups or (one-sample) compares the means of a group with a constant.

5.8 REFERENCES

1. Ragno G, Ioele G, De Luca M, Garofalo A, Grande F, Risoli A. A critical study on the application of the zero-crossing derivative spectrophotometry to the photodegradation monitoring of lacidipine. J Pharm Biomed Anal. 2006; 11: 42: 1: 39-45.
2. Corbella T R, Rodríguez D M A, Sanchez M J, Garcia M F. Comparative study of the zero-crossing, ratio spectra derivative and partial least-squares methods applied to the simultaneous determination of atrazine and its degradation product desethylatrazin-2-hydroxy in ground waters. Talanta. 1997; 44: 673 – 683.
3. Erk N. Quantitative analysis of chlorpheniramine maleate and phenylephrine hydrochloride in nasal drops by differential-derivative spectrophotometric, zero-crossing first derivative UV spectrophotometric and absorbance ratio methods. J.Pharm. Biom. Anal. 2000; 23: 1023-1031.
4. Dinc E, Kokdil G, Onur F. Spectrophotometric multicomponent analysis of mixture of metamizol, acetaminophen and caffeine in pharmaceutical formulations by two chemometric techniques. J Pharm Biom. Anal. 2001; 26: 969 - 978.
5. Dinc E, Baleanu D. A zero-crossing technique for the multi determination of thiamine HCl and pyridoxine HCl in their mixture by using one-dimensional wavelet transform. J Pharm Bio Anal. 2003; 31: 969 – 978.
6. Karljickovic R K, Novovic D, Marinkovic V, Agbaba D. First-order UV-derivative spectrophotometry in the analysis of omeprazole and pantoprazole

- sodium salt and corresponding impurities. *J Pharm Biom Anal.* 2003; 32: 1019 - 1027.
7. Nevado J J B, Flores J R, Pardo M L M, Fresenius. Recent Developments of Derivative Spectrophotometry and Their Analytical Applications. *J Anal Chem.* 1994; 349 – 756.
 8. Salinas F, Berzas N J J, Espinosa M A. A new spectrophotometric method for quantitative multicomponent analysis resolution of mixtures of salicylic and salicyluric acids. *Talanta.* 1990; 37: 347.
 9. Martin E, Hernandez O, Jimenez F, Arias J J. Spectrophotometric quantitative resolution of hydrochlorothiazide and spironolactone in tablets by chemometric analysis methods. *Anal. Chim. Acta.* 2001.
 10. Berzas J J, Guiberteau C C, Salinas F. Simultaneous determination of copper, cadmium and nickel by ratio derivative polarography. *Talanta.* 1992; 39: 547.
 11. Morelli B. Simultaneous determination of ceftriaxone and streptomycin in mixture by 'ratio-spectra' 2nd derivative and 'zero-crossing' 3rd derivative spectrophotometry. *Talanta.* 1994; 41: 673.
 12. Feyyaz O, Cem Y, Saadet D , Murat K, Gamze K. Simultaneous determination of pseudoephedrine sulfate, dextbrompheniramine maleate and loratadine in pharmaceutical preparations using derivative spectrophotometry and ratio spectra derivative spectrophotometry. *Talanta.* 2000; 51: 269.
 13. Nevin E. Spectrophotometric Analysis of Valsartan and Hydrochlorothiazide. *Anal. Lett.* 2002; 35: 2: 283 – 302.
 14. Davidson A G. Quantitative analysis of chlorpheniramine maleate and phenylephrine hydrochloride in nasal drops by differential-derivative spectrophotometric, zero-crossing first derivative UV spectrophotometric and absorbance ratio methods. *J Pharm Sci.* 1984; 73: 1582.
 15. Davidson A G, Stenlake J B. Assay of ephedrine hydrochloride and theophylline in pharmaceutical formulations by differential-derivative spectrophotometry. *Analyst.* 1974; 99: 476.
 16. USP XXII, United States Pharmacopoeia, 22nd revision, Convention Inc, Rockville, MD. 1990, 809.
 17. Moffat A C. Clarke's Isolation and Identification of Drugs, second ed, the pharmaceutical society of great Britain, London 1986.

- 18 . Curczak E W, Drennen J K. III Pharmaceutical and medical applications of near Infrared spectrophotometry, Marcel Dekker, Inc., New York. 2001; 73-105.
- 19 . McClure W F. Analysis using Fourier transform, In: D. A. Burns and E. W. Ciurczak, Editors, Handbook of near-Infrared Analysis, Marcel Dekker, Inc., New York. 1992; 181 -274.
- 20 . Garrigues S, Gallignani M, Guardia M D L. Simultaneous determination of orthometa and para-xylene by flow injection Fourier transform infrared spectrophotometry. Analyst. 1992; 117: 1849 – 1853.
- 21 . Miller B E, Danielson N D, Katon J E. Aqueous infra-red pharmaceutical analysis of two choline compounds by flow injection analysis using the CIRCLE [cylindrical internal reflectance] cell. Appl. Spectrosc. 1998; 42: 401 - 405.
- 22 . Munson J W. High-performance liquid chromatography; Theory: Instrumentation and pharmaceutical applications. In; Munson, J W, Eds. Pharmaceutical analysis modern methods: Part B, New York, Marcel Dekker, Inc. 2001; 155-156.
- 23 . Munson J W. Quantitative thin-layer chromatography. In; Munson, J W, Eds. Pharmaceutical analysis modern methods: Part B, New York, Marcel Dekker, Inc., 2001: 155-156.
- 24 . Maissa Y S, Mohamed G E B, Mohamed F E T, Eman S E Z. Simultaneous determination of domperidone maleate and cinnarizine in a binary mixture using derivative ratio spectrophotometry and classical least squares calibration J. Pharma. Biom. Ana. 2002; 30: 1: 21-33.
- 25 . Nevin E. Chemometric and derivative methods as flexible spectrophotometric approaches for dissolution and assaying tests in multicomponent tablets Analysis of binary mixtures of losartan potassium and hydrochlorothiazide by using high performance liquid chromatography, ratio derivative spectrophotometric and compensation technique. J. Pharma. Biom. Ana. 2001; 24: 603-611.
- 26 . Hector C G, Alejandro C O. Determination of bromhexine in cough-cold syrups by absorption spectrophotometry and multivariate calibration using partial least-squares and hybrid linear analyses Application of a novel method of wavelength selection. Talanta. 1999; 49: 793-800.

27. Zahra R, Bahram H, Soghra K, Mina G. Simultaneous spectrophotometric determination of carbamazepine and phenytoin in serum by PLS regression and comparison with HPLC. *Talanta*. 2005; 65: 21-28.
28. Erdal D, Ozgur U. Spectrophotometric quantitative resolution of hydrochlorothiazide and spironolactone in tablets by chemometric analysis methods. *II Farmaco*. 2003; 58: 1151- 1161.
29. Patricia C D, Andrea C M, Adrian J R, Fernando B, Alejandro C O. Design and optimization of a chemometrics-assisted spectrophotometric method for the simultaneous determination of levodopa and carbidopa in pharmaceutical products *Analytica Chimica Acta*. 2005; 543: 192-198.
30. Mnica C F, Ferraro P M, Castellano T S K. Chemometric determination of amiloride hydrochloride, atenolol, hydrochlorothiazide and timolol maleate in synthetic mixtures and pharmaceutical formulations. *J. Pharma. Biom. Ana.* 2004; 34: 305-314.
31. Prdal D, Ozgur U. Chemometric resolution of a mixture containing hydrochlorothiazide and amiloride by absorption and derivative spectrophotometry. *J. Pharma. Biom. Ana.* 2002; 29: 371-379
32. Alberto J N, Patricia C D, Alejandro C O. Chemometrics assisted spectrophotometric determination of vitamin B₆, vitamin B₁₂ and dexamethasone in injectables. *J. Pharma. Biom. Ana.* 2003; 3: 621- 627.
33. Albert R, Horwitz W. Validation of analytical procedure. *Anal. Chm.* 1997; 69: 789.
34. The United State Pharmacopoeia, 24th Edn., U S Pharmacopoeia Convention, Rockville, MD, 2000; 1923.
35. ICH, Q2B Validation of Analytical Procedure: Methodology, in: *Proceeding of the Imitational Conference: on Harmonization*, Geneva, March 1996.
36. ICH, Guidance on Analytical Method Validation, in: *Proceedings of International Convention on Quality for the Pharmaceutical Industry*, Toronto, Canada, September 2002.
37. ICH, Q1A Stability indicating method development and validation of Analytical Procedure: Methodology, in: *Proceeding of the Imitational Conference: on Harmonization*, Geneva, March 1996.