1. INTRODUCTION

1.1 General Introduction

Analytical methods are used for product research, product development, process control and chemical quality control purposes. Each of the techniques used in chromatographic or spectroscopic, have their own special features and deficiencies, which must be considered. Each step in the method must be investigated to determine the extent to which environment, matrix, or procedural variables can affect the estimation of analyte in the matrix from the time of collection up to the time of analysis [1-3].

Pharmaceutical analysis require very precise and accurate assay methods to quantify drugs either in Pharmaceutical or biological samples. The assay methods have to be sensitive, selective, rugged and reproducible [3].

Analytical chemistry is the qualitative and quantitative analysis of drug substances in biological fluids (mainly plasma and urine) or tissue. It plays a significant role in the evaluation and interpretation of pharmacokinetic data [2]. The main analytical phases comprise method development, method validation and sample analysis (method application).

1.1.1 Need for Pharmaceutical Analysis

- New Drug Development
- Method Validation as for ICH Guidelines
- Research in Pharmaceutical Sciences
- Clinical Pharmacokinetic Studies

When promising results are obtained from explorative validation performed during the method development phase, then only full validation should be stared. The process of validating a method cannot be separated from the actual development of method conditions.

1.1.2 Assay of Drugs and their Metabolites

A number of allusions have been made to analytical methods that distinguish drugs from their metabolites. Drug metabolism reactions can be divided into phase I and phase II categories. Phase I typically involves oxidation, reduction, and hydrolysis reactions. In contrast, phase II transformations involve coupling or condensation of drugs or their phase I metabolites with common body constituents.
(e.g., sulfate, glucuronic acid). Except for reduction processes, most phase I and phase II reactions yield metabolites that are more polar and hence more water soluble than the parent drug. Assays must distinguish between drug and its metabolites[5].

1.1.3 Analysis of Drugs from various samples

The most common samples obtained for pharmaceutical analysis are blood and urine. Feces are also utilized, especially if the drug or metabolite is poorly absorbed or extensively excreted in the bile. Other media that can be utilized include saliva, breath, and tissue.

Detection of a drug or its metabolite in biological media is usually complicated by the matrix. Because of this, various types of clean up procedures involving techniques such as solvent extraction and chromatography are employed to effectively separate drug components from endogenous material [6].

1.2 Extraction Procedures for Drugs and Metabolites from Biological Samples

After pretreating biological material, the next step is usually the extraction of the drugs from the biological matrix. All separation procedures use one or more treatments of matrix-containing solute with some fluid [6-8]. Different extraction procedures include protein precipitation or denaturation, liquid-liquid extraction, solid phase extraction and dehydration methods.

1.2.1 Protein Precipitation or Denaturation

Biological materials such as plasma, feces, and saliva contain significant quantities of protein, which can bind a drug. The drug should be free from this protein before further manipulation. Protein denaturation is important, because the presence of proteins, lipids, salts, and other endogenous material in the sample can cause rapid deterioration of HPLC columns and also interfere with the assay.

Protein denaturation procedures include the use of tungstic acid, ammonium sulfate, heat, alcohol, trichloroacetic acid and perchloric acid.

Methanol and acetonitrile frequently have been used as protein denaturants of biological samples. Methanol sometimes is preferred because it produces a flocculent precipitate and not the gummy mass obtained with acetonitrile. Methanol also gives a clear supernate and may prevent the drug entrapment that can be observed after acetonitrile precipitation.
1.2.2 **Liquid-Liquid Extraction**

Liquid-liquid extraction is the most widely used technique because

- The analyst can remove a drug or metabolite from larger concentrations of endogenous materials that might interfere with the final analytical determination.
- The technique is simple, rapid, and has a relatively small cost factor per sample.
- The extract containing the drug can be evaporated to dryness, and the residue can be redissolved in a smaller volume of a more appropriate solvent. In this manner, the sample becomes more compatible with a particular analytical methodology in the measurement step, such as a mobile phase in HPLC determinations.
- The extracted material can be redissolved in small volumes (e.g., 50 to 500 µl of solvent), thereby extending the sensitivity limits of an assay.
- It is possible to extract more than one sample concurrently.
- Near quantitative recoveries (90% or better) of most drugs can be obtained through multiple or continuous extractions.

1.2.3 **Solid Phase Extraction**

Liquid-solid extractions occur between a solid phase and a liquid phase. Among the solids that have been used successfully in the extraction (usually via adsorption) of drugs from liquid samples are XAD-2 resin, charcoal, alumina, silica gel and aluminum silicate. Sometimes the drugs are contained in a solid phase, such as in lyophilized specimens. Liquid-solid extraction is often particularly suitable for polar compounds that would otherwise tend to remain in the aqueous phase. The method could also be useful for amphoteric compounds that cannot be extracted easily from water.

Factors governing the adsorption and elution of drugs from the resin column include solvent polarity, flow rate of the solvent through the column, and the degree of contact the solvent has with the resin beads.

In the adsorption process, the hydrophobic portion of the solute that has little affinity for the water phase is preferentially adsorbed on the resin surface while the hydrophilic portion of the solute remains in the aqueous phase. Alteration in the
lipophilic / hydrophilic balance within the solute or solvent mix and not within the resin affects adsorption of the solute.

1.3 Method Development and Validation

1.3.1 Method Development

Method development involves evaluation and optimization of the various stages of sample preparation, chromatographic separation, detection and quantification.

Prior to method development of selected drug it is important for extensive literature survey regarding:

1. Choice of the instrument which is suitable for the analyte such as
   - Gas Chromatography (GC)
   - High Pressure Liquid Chromatography (HPLC)
   - Combined GC and LC Mass Spectrometry (GCMS)
   - HPLC-MS
   - LC-MS-MS
     - Choice of the mass parameters such as parent ion, product ion.
     - Choice of the ionization mode such as positive mode or negative.
     - Choice of the compound parameters such as DP, FP, CE and CXP.
     - Choice of the gas parameters such as curtain gas, nebulizer gas, heater gas and CAD gas

2. Choice of the chromatographic conditions such as
   - Mobile Phase, Column, Autosampler conditions
   - Flow rate, injection volume

3. Choice of the internal standard.

4. Choice of extraction method.

5. Choice of regression methods.

The method development and establishment for a analytical method include determination of selectivity, accuracy, precision, recovery, calibration curve, and stability of analyte in spiked samples [12].

Selectivity

It 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). Potential interfering substances in a biological matrix include endogenous matrix components, metabolites, decomposition products, and in the actual study, concomitant medication and other exogenous xenobiotics. If the method is intended to quantify more than one analyte, each analyte should be tested to ensure that there is no interference.

**Accuracy**

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. Accuracy should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The mean value should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%. The deviation of the mean from the true value serves as the measure of accuracy.

**Precision**

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 should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV. Precision is further subdivided into:

i. *Within-run or intra-batch precision*: This assesses precision during a single analytical run.

ii. *Between-run or inter-batch precision*: This measures precision with time, and may involve different analysts, equipment, reagents, and laboratories.
Recovery

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.

Calibration/Standard Curve

A calibration (standard) curve [3,4] is the relationship between instrument response and known concentrations of the analyte. It should be generated for each analyte in the sample. A sufficient number of standards should be used to adequately define the relationship between concentration and response. It 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. The number of standards used in constructing a calibration curve will be a function of the anticipated range of analytical values and the nature of the analyte/response relationship. Concentrations of standards should be chosen on the basis of the concentration range expected in a particular study.

A calibration curve should consist of
i. A blank sample (matrix sample processed without internal standard)
ii. A zero sample (matrix sample processed with internal standard)
iii. Six to eight non-zero samples covering the expected range, including LLOQ.

Lower Limit of Quantification (LLOQ)

The lowest standard on the calibration curve should be accepted as the limit of quantification if the following conditions are met:

- The analyte response at the LLOQ should be at least 5 times the response compared to blank response.
- Analyte peak (response) should be identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80-120%.
Stability in a Biological Fluid

Drug stability in a biological fluid [3,4,10,12] 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 should not be extrapolated to other matrices and container systems. Stability procedures should 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 should 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 should use 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 should be prepared in an appropriate solvent at known concentrations.

Freeze and Thaw Stability

Analyte stability should be determined after three freeze and thaw cycles. At least three aliquots at each of the low and high concentrations should be stored at the intended storage temperature for 24 hours and thawed unassisted at room temperature. When completely thawed, the samples should be refrozen for 12 to 24 hours under the same conditions. The freeze-thaw cycle should be repeated two more times, and then analyzed on the third cycle. If an analyte is unstable at the intended storage temperature, the stability sample should be frozen at -70°C during the three freeze and thaw cycles.

Short-Term Temperature Stability

Three aliquots of each of the low and high concentrations should be 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.
Long-Term Stability

The storage time in a long-term stability evaluation should exceed the duration between the date of first sample collection and the date of last sample analysis. Long-term stability should be 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 should be sufficient for analysis on three separate occasions. The concentrations of all the stability samples should be compared to the mean of back-calculated values for the standards at the appropriate concentrations from the first day of long-term stability testing.

Stock Solution Stability

The stability of stock solutions of drug and the internal standard should be evaluated at room temperature for at least 6 hours. If the stock solutions are refrigerated or frozen for the relevant period, the stability should be documented. After completion of the desired storage time, the stability should be tested by comparing the instrument response with that of freshly prepared solutions.

Post-preparative Stability/Autosampler Stability

The stability of processed samples, including the resident time in the autosampler, should be determined. The stability of the drug and the internal standard should be assessed over the anticipated run time for the batch size in validation samples by determining concentrations on the basis of original calibration standards. Other statistical approaches based on confidence limits for evaluation of analyte stability in a biological matrix can be used.

1.3.2 Method Validation

Method Validation [3,4,10,12] involves documenting, through the use of specific laboratory investigations, that the performance characteristics of the method are suitable and reliable for the intended analytical applications. The acceptability of analytical data corresponds directly to the criteria used to validate the method.

1.4 Estimation of Drugs In Biological Sample By LC-MS/MS

Most of the drugs in biological sample can be analysed by LC-MS/MS method because of several advantages like rapidity, specificity, accuracy, precision, ease of automation, eliminates tedious extraction and isolation procedures [19]. Some of the advantages are:
• Speed (analysis can be accomplished in 10 minutes or less)
• Greater sensitivity (various detectors can be employed)
• Improved resolution (wide variety of stationary phases)
• Reusable columns (expensive columns but can be used for many samples).
• Ideal for the substances of low volatility.
• Easy sample recovery, handling and maintenance.
• Instrumentation provides itself to automation and quantitation (less time).
• Precise and reproducible.
• Calculations are done by integrator itself.
• Suitable for preparative liquid chromatography on a much large scale.

There are different modes of separation in LC-MS. They are:
• Normal phase mode.
• Reverse phase mode.
• Reverse phase ion pair chromatography.
• Ion-Exchange chromatography.
• Affinity chromatography.
• Size Exclusion chromatography (gel permeation and gel filtration chromatography).

1.5. **Bioavailability and Bioequivalence Studies**

1.5.1 **Bioavailability Studies**

Bioavailability is defined as "the rate and extent to which the active ingredient or active moiety is absorbed from a drug product and becomes available at the site of action. For drug products that are not intended to be absorbed into the bloodstream, bioavailability may be assessed by measurements intended to reflect the rate and extent to which the active ingredient or active moiety becomes available at the site of action." This definition focuses on the processes by which the active ingredients or moieties are released from an oral dosage form and move to the site of action [20].

Bioavailability studies provide pharmacokinetic information related to the effects of the drug absorption, distribution and elimination, dose proportionality, linearity in pharmacokinetics of the active moieties and, inactive moieties.
Systemic exposure patterns reflect both release of the drug substance from the drug product and a series of possible presystemic/systemic actions on the drug substance after its release from the drug product[20].

The systemic exposure profiles of clinical trial material can be used as a benchmark for subsequent formulation changes and may thus be useful as a reference for future bioequivalence studies.

1.5.2. Pharmacokinetic Studies

The statutory definitions of BA, expressed in terms of rate and extent of absorption of the active ingredient or moiety to the site of action, emphasize the use of pharmacokinetic measures in an accessible biological matrix such as blood, plasma, and/or serum to indicate release of the drug substance from the drug product into the systemic circulation.

Both direct (e.g., rate constant, rate profile) and indirect (e.g., $C_{\text{max}}, T_{\text{max}}$, mean absorption time, mean residence time, $C_{\text{max}}$ normalized to AUC). Parameters on systemic exposure measures should reflect comparable rate and extent of absorption, which in turn should achieve the underlying statutory and regulatory objective of ensuring comparable therapeutic effects. Exposure measures are defined relative to early exposure, peak exposure, and total exposure portions of the plasma, serum, or blood concentration time profile [10].

The pharmacokinetic, pharmacodynamic, clinical, and in vitro studies can be used to measure product quality [11-12].