www.pharmaerudition.org

ISSN: 2249-3875

International Journal of Pharmaceutical Erudition

Research for Present and Next Generation

AUG 2019

Vol: 09 Issue:02

(24-44)

ISSN 2249-3875

International Journal of Pharmaceutical Erudition

Review Article

BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF PRIMIDONE IN K3EDTA HUMAN PLASMA BY USING LC-MS/MS

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Bioanalytical method development is the process of creating a procedure to enable a compound of interest to be identified and quantified in a biological matrix. A compound can often be measured by several methods and the choice of analytical method involves many considerations. Analysis of drugs and their metabolites in a biological matrix is carried out using different extraction techniques like liquid-liquid extraction, solid phase extraction (SPE) and protein precipitation from these extraction methods samples are spiked with calibration (reference) standards and using quality control (QC) samples. These methods and choice of analytical method describes the process of method development and includes sampling, sample preparation, separation, detection and evaluation of the results. The developed process is then validated. These Bioanalytical validations play a significant role in evaluation and interpretation of bioavailability, bioequivalence, pharmacokinetic, and toxicokinetic studies. In which different parameters like accuracy, precision, selectivity, sensitivity, reproducibility, and stability are performed.

Key Words: -LLE, SPE, Quality control samples, Bioequivalence, Bioavailability, Validation.

INTRODUCTION

Bio-Analytical Method

The methods employed for detection and measurement of drug concentrations in biological fluids are referred to as Bio-Analytical Methods. Bio-analytical methods are widely used to quantitate drugs and their metabolites in physiological matrices.

Need of Bio-Analytical Method

- To investigate the pharmacokinetic of new drug candidates.
- To compare pharmacokinetic profiles of different formulations.
- To monitor drug levels to establish the appropriate dose or frequency of administration.
- For fast and reliable measurement of the compounds in biological matrices.
- A bio-analytical method consists of two main components:

Sample Preparation

Determination of drug and its metabolites

- Sample preparation is a method used to clean up a sample before analysis and to concentrate a sample to improve its detection.
- Sample preparation in the Bio-analysis: it is the “last frontier” and starting point for the accurate LC-MS/MS analysis.
- Processing step includes;
  - Method Development
  - Validation

- Assay Performance and
- Work Flow.

➢ Criteria for Bio-analytical method;

- Simplicity of the developed method
- Short Method Development time and
- Validation failures kept at the minimum

➢ Develop the most selective sample preparation method.

How to develop Bioanalytical Method:-

- Peak Plasma Concentration
- Physicochemical Property
- Determination of ULOQ, LLOQ
- Selection of drug volume to be spiked
- Sample preparation

➢ **Peak Plasma Concentration (Cmax):** In Cmax, maximum concentration of analyte in biological fluid to be determined from literature survey, which is helps in establishment of target sensitivity of the developing method.

➢ **Physicochemical Property:** Physicochemical Properties of an analyte of interest such as solubility, molecular weight, structure, melting point, dissociation constant (pKa) which is helps in selection of the suitable extraction method.

➢ **Determination of Lower and Upper Limit of Quantification:** The lowest concentration of an analyte in a sample that can be quantitatively determined with an acceptable precision and accuracy is usually 1/20th ^{of} the Cmax value. After calculating ULOQ and LLOQ value have to prepare standard stock solution from which solution of different concentration are prepared.

➢ **Selection of drug volume to be spiked:** The volume of analyte of interest is depends upon the volume of plasma spiked. Analyte concentration is normally 5% of the spiked plasma volume. For Example; if spiked plasma volume is 500µL, so the volume of analyte to be added will be 25µL.

➢ **Sample preparation:** Sample preparation technique is used to the clean up a sample by removing endogenous material as well as to concentrate a sample before analysis to exclude errors in its detection.

Variables used in Method Development:

- **Internal Standard-** "It is a different compound from the analyte but one that is well resolved in the separation. One of main reason for using an ISTD is for samples requiring significant pre-treatment or preparation. Often, sample preparation step that include reaction, filtration, result in sample losses. When added prior to sample precipitation, a properly chosen internal standard can be used to correct for these sample losses".
- With internal standard method, a calibration plot is produced by preparing and analyzing calibration solutions containing different concentration of compound of interest with a fixed concentration of internal standard added.
- **Requirements for a proper internal standard includes:**
- Well resolved from compound of interest and other peaks. Should not be in original

ISSN 2249-3875

sample.

- Should mimic the analyte in any sample preparation steps.
- Does not have to be chemically similar to analyte.
- Commercially available in high purity.
- Stable and un- reactive with sample or mobile phase. Should have a similar detector response to analyte for concentration used.
- As per EMA guidelines, when mass-spectrometry detection is used in the Bioanalytical method, a stable isotope-labelled ISTD is recommended to be used whenever possible. However, it is essential that the labelled standard is of the highest isotope purity. The presence of any unlabelled analyte should be checked and if relative amounts of unlabelled analyte are detected the potential influence has to be evaluated during method validation.
- **Buffering Agent-** In solvent extraction, ionic analytes often can be transferred into either phase, depending on the selected condition. If the aqueous phase is buffered at least 1.5 pH units above its pKa value, the analyte will be ionized and prefer aqueous phase, less polar interferences will be extracted into organic phase. If the pH of the aqueous solution is lowered, so that the analyte is no longer ionized, analyte will be extracted into the organic phase, leaving more polar interferences in the aqueous phase.
- **Mobile Phase Buffer-** Wherever acidic or basic samples are separated it is strongly advisable to control mobile phase pH by adding a buffer. The measurement of pH for a mobile phase that contains organic solvent is imprecise, because electrode response tends to drift. Consequently if a pH is to be used, it is strongly recommended that pH of buffer should be adjusted before adding organic solvent. In selecting a particular buffer, several considerations should be kept in mind.
- Buffer capacity- is determined by pH, buffer pKa and buffer concentration. As for the case of a sample compound, buffer ionization occurs over a range in pH given by pKa ± 1.5. Only in this pH range, buffer can be effective in controlling pH. Therefore, to be on safe side, the buffer selected for a particular separation should be used to control pH over a range ≈ pKa ± 1. For RPC separation, a buffer concentration of 10- 50 mM is usually adequate. Higher buffer concentrations also may adversely affect the operation of HPLC systems constructed of stainless steel. A mobile phase with marginal buffer capacity will give less reproducible separations for compounds that are practically ionized at the pH of the mobile phase. In this case, retention may change from run to run, and distorted peaks may result.
- Buffer solubility and stability, possible interaction with equipment, sample, and / or

column, and the volatility are also of interest for some applications.

- Volatile buffers are useful for two kinds of applications. If purified sample components are to be recovered, it is convenient to be able to remove buffer by evaporation or lyophilisation. Buffers such as **Ammonium Carbonate, Ammonium Formate, Ammonium Acetate, and Trifluoroacetic acid** are useful in this regard.
- Volatile buffer may also be required for use with some detector such as light scattering or mass spectrometer.

Application of Bio-Analytical Method

- The determination of drug concentrations in biological fluids yields the data used to understand the time course of drug action, or pharmacokinetics, in animals and man and is an essential component of the drug discovery and development process.
- Bio-analysis, employed for the quantitative determination of drugs and their metabolites in biological fluids, plays a significant role in the evaluation and interpretation of Bioequivalence, Pharmacokinetic (PK), and Toxicokinetic studies.
- Selective and sensitive analytical methods for quantitative evaluation of drugs and their metabolites are critical for the successful conduct of pre-clinical and/or bio-pharmaceutics and clinical pharmacology studies.

Various Methods Used for Sample Preparation is as Follows

1. Solid Phase Extraction **(SPE)**
2. Liquid-Liquid Extraction **(LLE)**
3. Precipitation **(PPT)**

1. Solid Phase Extraction (SPE)

➢ Solid phase extraction (SPE) is the most widely used sample preparation technique in the analysis of new pharmaceutical compounds and metabolites in biological matrices.

➢ SPE employs a small plastic disposable column or cartridge, often the barrel of a medical syringe packed suitable sorbent.

➢ SPE utilizes four types of separations - reversed phase, normal phase, ion-exchange and adsorption based on interaction between analyte and sorbent. Analyte of interest may be either un-retained, while interferences are adsorbed or retained, while interferences are washed through.

➢ The first strategy is usually chosen when the desired sample component is present in high concentration. When components of interest are present at low levels, or multiple components of widely differing polarities need to be isolated, the second strategy is generally employed.

➢ The second strategy may also be used for trace enrichment of extremely low level compounds and concentration of dilute sample. A complex matrix may be treated by both elution strategies to isolate different target

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International Journal of Pharmaceutical Erudition

analyte.

❖ **Advantages of SPE:-**

- Faster sample preparation
- Lower cost- less solvent and reagent consumption and therefore less hazardous waste
- Greater recoveries- no cross contamination
- Less sample handling- no emulsion problem
- Reduced harm to labile samples- minimal evaporation
- Improved safety- due to reduced solvent/sample exposure and glassware
- Easy automation- simultaneous batch processing of multi-samples

➢ There are two SPE methods,

1. Off-line SPE
2. On-line SPE

1. Off-line SPE

Advantages

- Selective sample clean-up
- Various SPE formats – phases
- Sample pre-concentration
- Automated systems
- Trend to 96-well micro SPE (avoids drying steps)

Disadvantages

- Labour intensive method development
- Evaporation steps often needed
- Multiple steps, difficult sample traceability
- Risk of running dry Limited sample

1. Off-line SPE

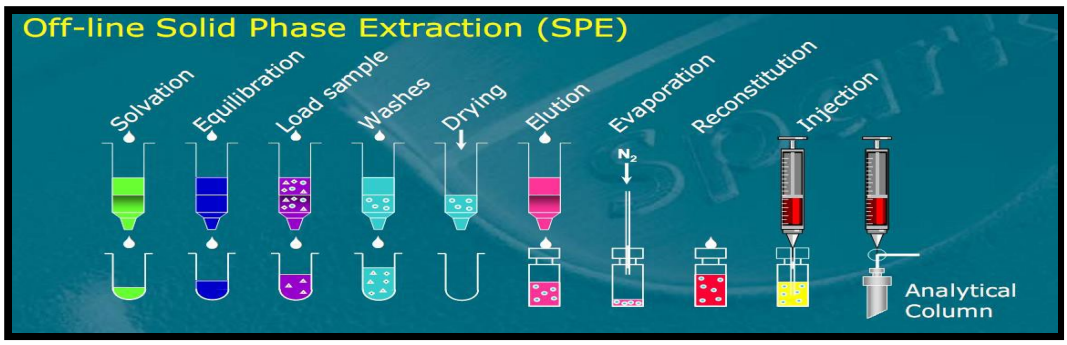

Fig. 1: Flow of off-line SPE method

2. On-line SPE

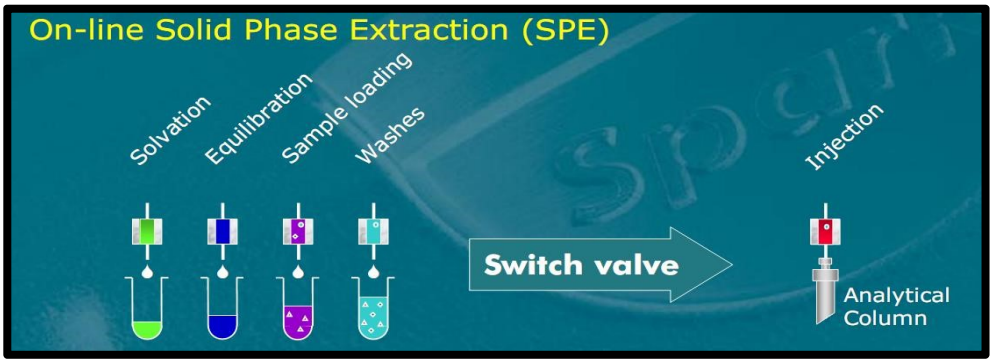

Fig 2: Flow of on-line SPE method

ISSN 2249-3875

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processing pressures Sample prep at day-time, not a fully automated process

❖ **Advantages:-**

- Disposable SPE cartridges
- Minimal pre-treatment steps
- Sample preparation at high pressure and controlled flow-rates
- All samples are analyzed, sensitive assays
- Systematic, automated method development
- Fully automated and integrated process

❖ **Disadvantages:-**

- Integrated sample prep system needs higher qualified and well trained operators
- Biological samples in the LC-MS lab
- Linked to one MS system, not a workstation approach

2. Liquid-Liquid Extraction (LLE)

➢ Liquid-liquid extraction is based on distribution of solutes between an aqueous phase and a water immiscible organic phase. Distribution of different solutes depends on their degree of solubility in different solvents.

➢ In bio-analytical application of liquid-liquid extraction, it is useful for separating analyte from interferences present in biological matrices.

➢ Analyte extracted into the organic phase can be evaporated to dryness and the residue reconstituted in a smaller volume of an appropriate solvent (preferably mobile phase), while analyte extracted in to the aqueous phase can often be injected directly on to a reversed-phase column.

➢ The technique is simple and rapid. Good quantitative recoveries are obtained through multiple continuous extractions.

❖ **Advantages:-**

- Known and standard technique
- Relatively simple to do as a routine
- Trend to micro LLE
- Low LODs are possible
- Cost effective as compare to the On-line and Off-line SPE techniques

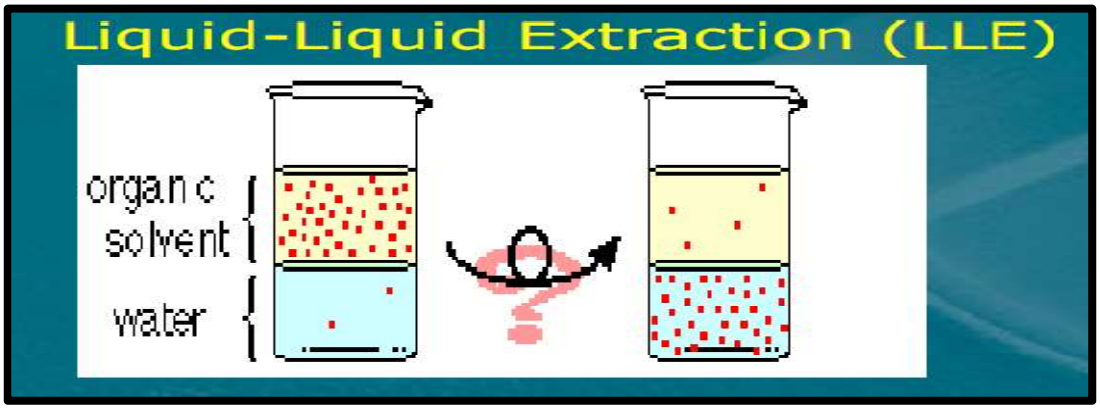

Fig. 3: LLE

Clean sample obtain as compare to Protein Precipitation Technique

❖ **Disadvantages:-**

- Moderate selective (limited in solvent selections)
- Examine and use pH/ionic strength/temperature to get a selective extraction process
- Multiple extractions needed to get the recovery
- Often evaporation steps needed
- Emulsions formed cause recovery loss
- Difficult to automate, semi-automated steps
- As routine method labour intensive
- Long method development time & Sample prep during day time only Limited amount of non-water soluble extraction solvents
- Examine the influence of pH, ionic strengths, temperature to shift equilibrium

➢ Solvents normally used are Ethyl Acetate, Tert Butyl methyl ether, Diethyl ether, n-Hexane and sometimes mixtures of the two or more solvent for efficient extraction.

3. Precipitation (PPT)

➢ Protein precipitation is the simple method of extraction as compared to the LLE and SPE. It is utilized when high throughput of plasma and serum samples is desired.

➢ Protein precipitation is very useful method in field of clinical toxicology, the drug discovery and the therapeutic drug monitoring in which high throughput is required.

➢ Proteins are denatured with acid, base, salts or organic solvents. (e.g., TFA, TCA, NaOH, ZnSO4, Acetonitrile, Methanol).

➢ Acetonitrile is the first choice of solvent for protein precipitation due to its complete precipitation of proteins and methanol is the second choice of organic precipitant provided the solubility of the analyte in these solvents.

➢ Now a day only used in bio-analysis for removal of proteins. It is only a sample preparation not an extraction method.

➢ After protein precipitation centrifugation is carried out and the supernatant obtained is used for analysis. The supernatant can be injected directly into the chromatographic column or it can be evaporated and reconstituted with the mobile phase.

❖ **Advantages:-**

- Simple, generic, easy to perform, just mix solvents - centrifuge/filtrate
- No Method Development time
- Fast sample preparation (96-well titer plate automation)
- Very low volume require for sample processing
- Very few step to get final drug concentrate ready for inject into LC-MS/MS

❖ **Disadvantages:-**

- Not selective, limited clean-up with a high potential of ion-suppression in LC-MS analysis

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- Drugs can precipitate with proteins
- Not selective (relatively dirty extracts)
- Centrifugation - filtration or additional clean-up steps are needed
- Sample is diluted, no pre-concentration steps/low sensitivity assays
- Proteins, peptides (< 5000 Da) are not precipitated
- Not sensitive (sample dilution + high background)
- Method Development and sample preparation during day time only
- Relatively high chances on validation & run failures
- Ion-suppression and Recovery losses

Various Biological Fluids Used are

✓ Blood
✓ Plasma
✓ Serum
✓ Urine
✓ Cerebro spinal fluid
✓ Tissue homogenizes and
✓ Saliva

Various Analytical Techniques Used for the Detection of Drug in Biological Fluid is

❖ **Hyphenated techniques**

1. LC-DAD (Liquid Chromatography - Diode Array Detection)
2. CE-MS (Capillary Electrophoresis - Mass Spectrometry)
3. LC-MS (Liquid Chromatography - Mass Spectrometry)
4. **LC-MS/MS (Tandem Mass Spectrometry)**
5. GC-MS (Gas Chromatography - Mass Spectrometry)

❖ **Chromatographic methods**

1. HPLC (High Performance Liquid Chromatography)
2. GC (Gas Chromatography)
3. UPLC (Ultra Performance Liquid Chromatography)
4. Supercritical Fluid Chromatography

❖ **Electrophoresis**

❖ **Ligand binding assays**

1. Dual Polarization Interferometry
2. ELISA (Enzyme-Linked Immunosorbent Assay)
3. MIA (Magnetic Immunoassay)
4. RIA (Radioimmunoassay)

❖ **Mass spectroscopy**

❖ **Nuclear magnetic resonance**

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pharmacokinetic (PK), and toxicokinetic studies.

- Selective and sensitive analytical methods for quantitative evaluation of drugs and their metabolites are critical for the successful conduct of pre-clinical and/or bio-pharmaceutics and clinical pharmacology studies.

BIO-ANALYTICAL METHOD VALIDATION

➢ Validation may be defined as documented evidence that provides a high degree of assurance that a specific process will consistently produce a product meeting its pre-determined specifications and quality attributes.

➢ Method validation is the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use.

Objective of Validation

- To yield reliable result
- To minimize the error
- To define the requirements for establishing & implementing an effective method
- To ensure that a particular method for quantitative measurement of an analyte in a biological matrix is reliable and reproducible

Different Types and Levels of Validation

1. Full Validation
2. Partial Validation
3. Cross Validation

1. Full Validation

➢ Full validation is important when developing and implementing a bio-analytical method for the first time. Full validation is important for a new drug entity.

➢ A full validation of the revised assay is important if metabolites are added to an existing assay for quantification.

➢ The objective of full validation is to demonstrate selectivity, lower limit of quantification (LLOQ), calibration curve, accuracy, precision, matrix effect, recovery, carry-over, dilution integrity, and stability.

➢ Generally, a full validation should be performed for each species and matrix (mainly plasma, serum, whole blood, or urine) to be analyzed.

➢ The matrix used in analytical validation should be as close as possible to the intended study samples, including anticoagulant and additives.

2. Partial Validation

➢ Partial validations are modifications of already validated bio-analytical methods.

➢ Partial validation may be performed when minor changes are made to an analytical method that has already been fully validated. It can range from as little as one intra-assay accuracy and precision determination to a nearly full validation.

➢ A set of parameters to be evaluated in a partial validation are determined according to the extent and nature of the changes made to the method.

- Typical bio-analytical method changes that fall into this category include, but are not limited to:
  - Bio-analytical method transfers between laboratories or analysts
  - Change in analytical methodology (e.g., change in detection systems)
  - Change in anticoagulant in harvesting biological fluid
  - Change in matrix within species (e.g., human plasma to human urine)
  - Change in sample processing procedures
  - Change in species within matrix (e.g., rat plasma to mouse plasma)
  - Change in relevant concentration range
  - Changes in instruments and/or software platforms
  - Limited sample volume (e.g., pediatric study)
  - Rare matrices
  - Selectivity demonstration of an analyte in the presence of concomitant medications
  - Selectivity demonstration of an analyte in the presence of specific metabolites
  - Method does not giving the response that it has to give means no method reproducibility due to certain problem

3. Cross Validation

- Cross-validation is a comparison of validation parameters when two or more bio-analytical methods are used to generate data within the same study or across different studies.
- An example of cross validation would be a situation where an original validated bio-analytical method serves as the reference and the revised bio-analytical method is the comparator. The comparisons should be done both ways.
- When sample analyses within a single study are conducted at more than one site or more than one laboratory, cross-validation with spiked matrix standards and subject samples should be conducted at each site or laboratory to establish inter laboratory reliability.
- Cross-validation should also be considered when data generated using different analytical techniques (e.g., LC-MS-MS vs. ELISA) in different studies are included in a regulatory submission.
- In the cross validation conducted after full or partial validation in each laboratory or for each analytical method to be compared, the same set of QC samples spiked with the analyte or the same set of study samples is analyzed at both laboratories or by both analytical methods, and the mean accuracy at each concentration level or the assay variability is evaluated.
- In the cross validation between different analytical methods, both validation procedure and acceptance criteria (i.e., acceptable assay variability) should be separately defined based on scientific judgment

by considering the nature of the analytical methods.

Parameter Use for Validation of the Bioanalytical Method

- Measurements for each analyte in the biological matrix should be validated. In addition, the stability of the analyte in spiked samples should be determined.
- 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.

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).

➢ 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, 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. 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.

➢ 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 3 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 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 un-extracted standards that represent 100 % recovery.

Calibration/Standard curve

➢ A calibration (standard) curve is the relationship between instrument response and known concentrations of the analyte. A calibration curve should be generated for each analyte in the 6 sample. A sufficient number of standards should be used to adequately define the relationship between concentration and response.

➢ 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. 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 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.

1. 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: C The analyte response at the LLOQ should be at least 5 times the response compared to blank response.

- **Criteria**: - Analyte peak (response) should be identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80 - 120 %.

ISSN 2249-3875

3. Calibration Curve/Standard Curve/Concentration-Response

➢ The simplest model that adequately describes the concentration-response relationship should be used. Selection of weighting and use of a complex regression equation should be justified. The following conditions should be met in developing a calibration curve:

➢ C #20% deviation of the LLOQ from nominal concentration.

➢ C #15% deviation of standards other than LLOQ from nominal concentration at least four out of six non-zero standards should meet the above criteria, including the LLOQ and the calibration standard at the highest concentration. Excluding the standards should not change the model used.

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 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.

1. 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 -700C during the three freeze and thaw cycles.

2. 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.

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 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.

4. 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.

5. Post-Preparative 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.

Dilution Integrity

➢ Dilution integrity is performed in order to check the validity of method in case the sample needs to be diluted during analysis.

It is done by spiking analyte working standard in drug free and interference free plasma to get concentration 4-5 times of ULOQ. Five and ten fold dilution made of the original concentration using screened and pooled plasma and analysed against a fresh calibration curve. The concentration will be calculated using the dilution factor.

HPLC

HPLC means High Pressure Liquid Chromatography or High Performance Liquid Chromatography. It is the most widely used analytical separation technique.

➢ Chromatography is a separation technique used to separate and quantify each component of the sample by

➢ passing the mixture through stationary phase and mobile phase.

➢ HPLC works on the principle of adsorption. When a sample solution is passed through stationary phase, different components of the sample travels with different velocity

ISSN 2249-3875

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Instrumentation

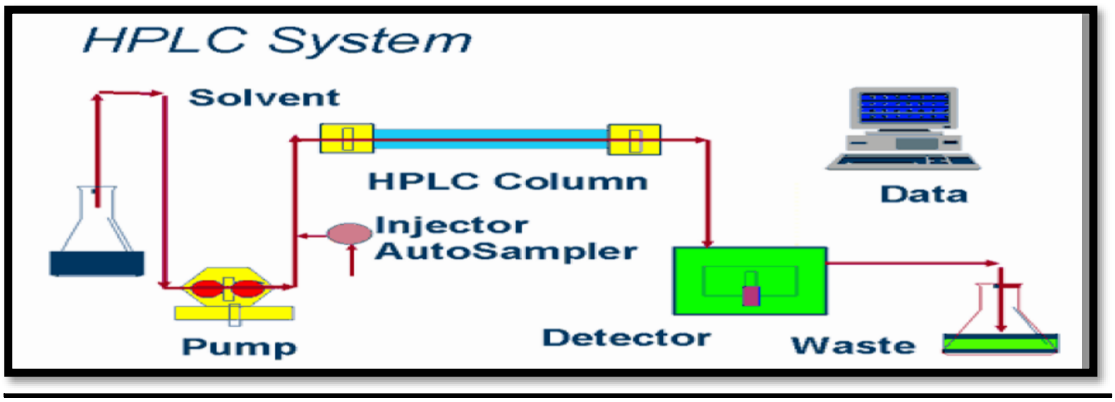

Fig. 4: Schematic diagram of HPLC

➢ . depending upon their affinity towards the stationary phase and eluted out.

Different Modes of Separation

- Normal Phase HPLC
- Reversed Phase HPLC

I. Solvent Reservoir and Degasser

Solvent reservoir contains the mobile phase and the sample solution. A degasser is needed to remove dissolved air and other gases from the solvent

II. Pumps

The pump is one of the most important components of HPLC, since its performance directly affects retention time, reproducibility and detector sensitivity. Three main types of pumps used in HPLC are:

i) Displacement pump

ii) Reciprocating pump

iii) Pneumatic or constant pressure pump

III. Sample Injector

There are three important ways of introducing the sample into injection port:

i) Loop injection

ii) Valve injection

iii) On column injection

IV. Column

Columns with an internal diameter of 5 mm give good results because of compromise between efficiency, sample capacity, and the amount of packing and solvent required.

- **Column Packing**

The packing used in modern HPLC consist of small, rigid particles having a narrow particle size distribution. There are three main types of column packing in HPLC:

i) Porous, polymeric beds

ii) Porous layer beds

iii) Totally Porous silica particles (diameter < 10 µ)

V. Detector

The function of the detector in HPLC is to monitor the mobile phase as it emerges from the column. Detectors are usually of two types:

i) Bulk property detectors: Ex. - refractive index, dielectric constant or density.

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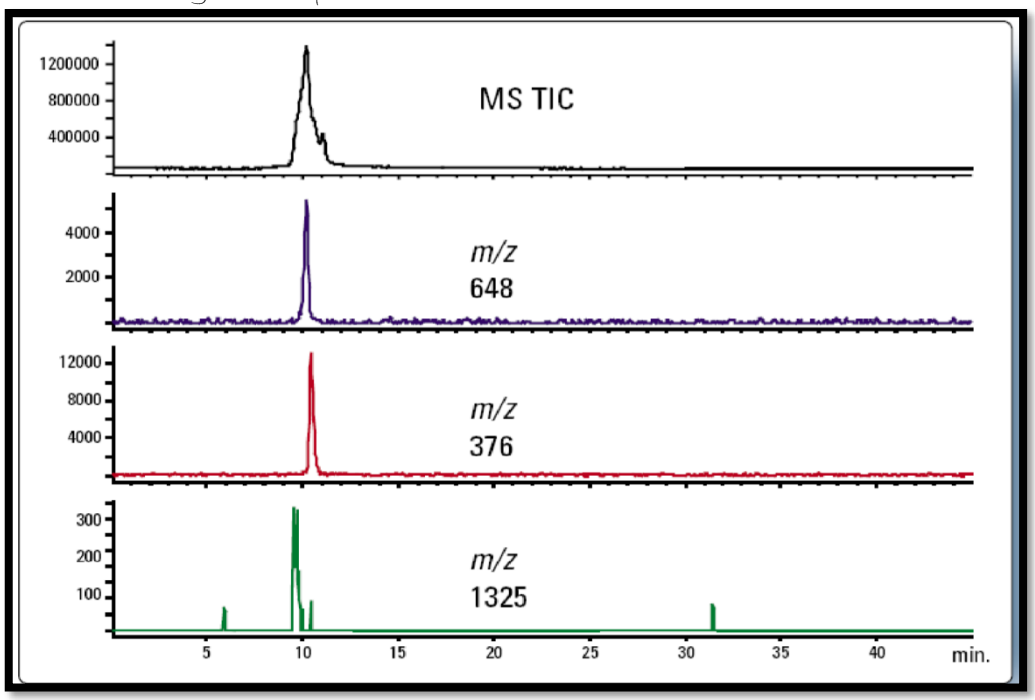

Fig.5: Identification of three components in a chromatographically

ii) Solute property detectors: Ex. - UV absorbance, fluorescence or diffusion current. Now a day solute property detectors use widely because of its high sensitivity compares to bulk property detectors.

Various Parameters Needs to be controlled are

1. Number of Theoretical plates
2. Column diameter, Particle size and temperature
3. Mobile phase composition
4. Pump pressure
5. Capacity factor
6. Selectivity factor

Application of HPLC

- Measuring the level of active drugs, synthetic by-product, and degradation product in pharmaceutical dosage forms.
- Separating polymers and determining the molecular weight distribution of polymer in the mixture.
- Separation and analysis of non-volatile or thermally unstable compounds.
- Measuring levels of certain compounds such as amino acid, nucleic acid, and proteins in physiological samples.

WHY LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY?

➢ Liquid chromatography is a fundamental separation technique in the life sciences and related fields of chemistry. Unlike gas chromatography, which is unsuitable for non-volatile and thermally fragile molecules, liquid chromatography can safely separate a very wide range of organic compounds, from small-molecule drug metabolites to peptides and proteins.

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➢ Traditional detectors for liquid chromatography include refractive index, electrochemical, fluorescence, and ultraviolet-visible (UV-Vis) detectors.

➢ For most compounds, mass spectrometers are more sensitive and far more specific than all other LC detectors. It can analyze compounds that lack a suitable chromophore. It can also identify components in unresolved chromatographic peaks, reducing the need for perfect chromatography.

➢ There are certain compounds that's have the same UV spectra that can be identify, confirm, and quantify using the mass spectrometer.

➢ Some mass spectrometers have the ability to perform multiple steps of mass spectrometry on a single sample. They can generate a mass spectrum, select a specific ion from that spectrum, fragment the ion, and generate another mass spectrum; repeating the entire cycle many times. Such mass spectrometers can literally deconstruct a complex molecule piece by piece until its structure is determined.

➢ Some mass spectrometers have the ability to perform multiple steps of mass spectrometry on a single sample. They can generate a mass spectrum, select a specific ion from that spectrum, fragment the ion, and generate another mass.

➢ Spectrum; repeating the entire cycle many times. Such mass spectrometers can literally deconstruct a complex molecule piece by piece until its structure is determined.

Instrumentation

➢ Mass spectrometers work by ionizing molecules and then sorting and identifying the ions according to their mass-to-charge (*m/z*) ratios. Two key components in this process are the ion source, which generates the ions, and the mass analyzer, which sorts the ions.

Ion sources

- Earlier LC/MS systems used interfaces that either did not separate the mobile phase molecules from the analyte molecules (direct liquid inlet, thermo spray) or if did so before ionization (particle beam). The analytes were then ionized under vacuum, often by traditional electron ionization. These approaches were successful only for a very limited number of compounds.
- In atmospheric pressure ionization, the analyte molecules are ionized first, at atmospheric pressure. The analyte ions are then mechanically and electro-statically separated from neutral molecules. Common atmospheric pressure ionization techniques are:

I. Electrospray Ionization (ESI)

II. Atmospheric Pressure Chemical Ionization (APCI)

III. Atmospheric Pressure Photoionization (APPI)

❖ **Electrospray Ionization**

Electrospray relies in part on chemistry

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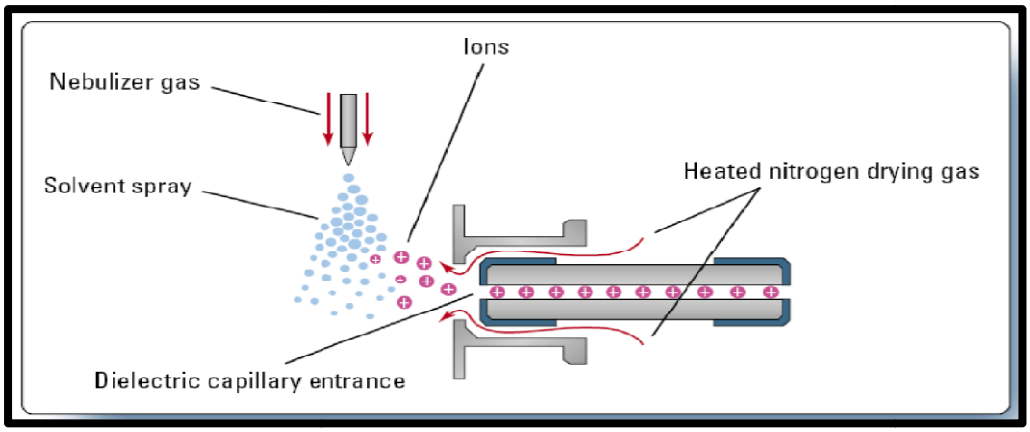

Fig.6: Electrospray ion source

to generate analyte ions in solution before the analyte reaches the mass spectrometer. The LC eluent is sprayed (nebulized) into a chamber at atmospheric pressure in the presence of a strong electrostatic field and heated drying gas.

➢ The electrostatic field causes further dissociation of the analyte molecules. The heated drying gas causes the solvent in the droplets to evaporate. As the droplets shrink, the charge concentration in the droplets increases. Eventually, the repulsive force between ions with like charges exceeds the cohesive forces; ions are ejected (desorbed) into the gas phase. These ions are attracted to and pass through a capillary sampling orifice into the mass analyzer.

Electrospray is especially useful for analyzing large biomolecules such as proteins, peptides, and oligonucleotides, but can also analyze smaller molecules like benzodiazepines and sulfated conjugates.

Mass analyzers

➢ Four types of mass analyzer used for LC/MS:

I. Quadrupole

II. Time - Of - Flight

III. Ion Trap

IV. Fourier Transform - Ion Cyclotron Resonance (FT-ICR or FT-MS)

➢ Each has advantages and disadvantages depending on the requirements of a particular analysis.

❖ **<u>Quadrupole</u>**

➢ A quadrupole mass analyzer consists of four parallel rods arranged in a square. The analyte ions are directed down the center of the square. Voltages applied to the rods generate electromagnetic fields. These fields determine which mass-to-charge ratio of ions can pass through the filter at a given time.

➢ Quadrupole mass analyzers can operate in two modes:

- Scanning (scan) mode
- Selected Ion Monitoring (SIM) mode

➢ In scan mode, the mass analyzer

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monitors a range of mass-to-charge ratios. In SIM mode, the mass analyzer monitors only a few mass to charge ratios. Scan mode is typically used for qualitative analyses or for quantitation when all analyte masses are not known in advance. SIM mode is used for quantitation and monitoring of target compounds.

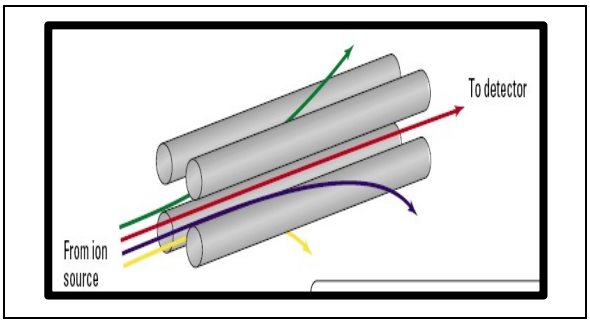

Fig. 7: Quadrupole mass analyzer

Collision-Induced Dissociation and Multiple Stage MS

➢ The atmospheric pressure ionization techniques discussed are all relatively "soft" techniques. They generate primarily:

- Molecular ions M^+ or M^-
- Protonated molecules $[M + H]^+$
- Simple adduct ions $[M + Na]^+$
- Ions representing simple losses such as the loss of a water $[M + H - H2O]^+$

➢ The resulting molecular weight information is very valuable, but complementary structural information is often needed. To obtain structural information, analyte ions are fragmented by colliding them with neutral molecules in a process known as collision induced dissociation (CID) or collision all activated dissociation (CAD). Voltages are applied to the analyte ions to add energy to the collisions and create more fragmentation.

I. CID in single-stage MS

- CID is most often associated with multistage mass spectrometers where it takes place between each stage of MS filtering, but CID can also be accomplished in single-stage quadrupole or time-of-flight mass spectrometers.
- In single-stage mass spectrometers, CID takes place in the ion source and is thus sometimes called source CID or in-source CID. Analyte (precursor) ions are accelerated and collide with residual neutral molecules to yield fragments called product ions.
- The advantage of performing CID in single stage instruments is their simplicity and relatively low cost. The disadvantage is that all ions present are fragmented.
- The resulting spectra may include mass peaks from background ions or co-eluting compounds as well as those from the analyte of interest. This trade-off may be acceptable when analyzing relatively pure samples, but does not give good results if chromatographic peaks are not well resolved or background levels are high.

II. CID and multiple-stage MS

- Multiple-stage MS (also called tandem MS or MS/MS or MSn) is a powerful way to obtain structural information.

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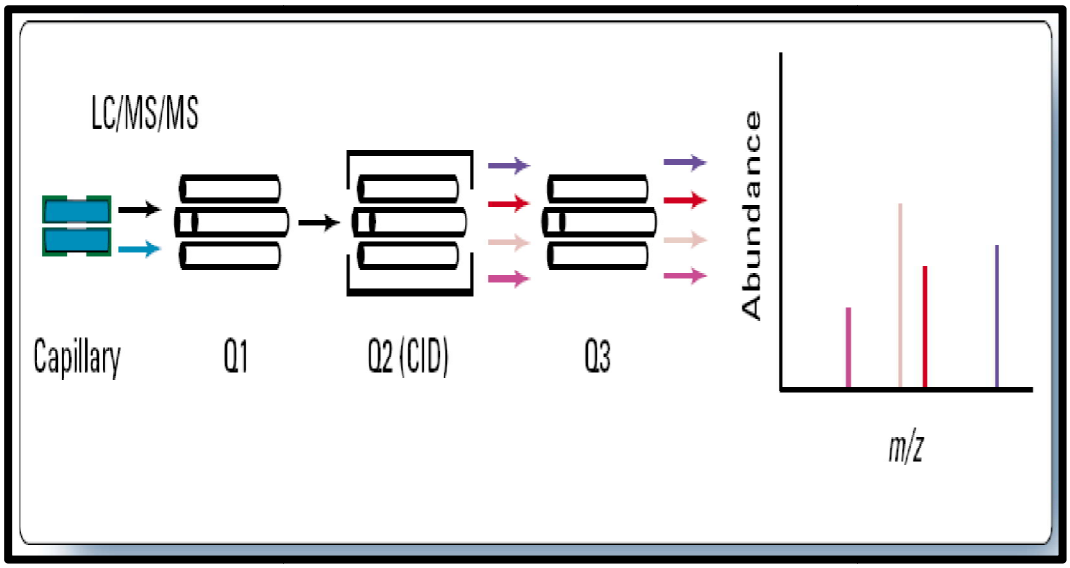

Fig.8: MS/MS in triple quadrupole mass spectrometer

- In triple-quadrupole or quadrupole/quadrupole/time-of-flight instruments (see Figure 1.5); the first quadrupole is used to select the precursor ion. CID takes place in the second stage (quadrupole or octapole), which is called the collision cell.

The third stage (quadrupole or TOF) then generates a spectrum of the resulting product ions. It can also perform selected ion monitoring of only a few product ions when do quantitation of target compounds.

In ion trap and FT-ICR mass spectrometers, all ions except the desired precursor ion are ejected from the trap. The precursor ion is then energized and collided to generate product ions. The product ions can be ejected to generate a mass spectrum, or a particular product ion can be retained and collided to obtain another set of product ions

Application of LC - MS/MS

- **Molecular Weight Determination**

- Differentiation of similar octapeptides

- **Structural Determination**

- Structural determination of ginsenosides using MSn analysis

- **Pharmaceutical Applications**

- Rapid chromatography of benzodiazepines

- Identification of degradation products for salbutamol and bile acid metabolites

- **Biochemical Applications**

- Rapid protein identification using capillary LC/MS/MS & database searching

- **Clinical Applications**

- High-sensitivity detection of trimipramine and thioridazine

- **Food Applications**

- Identification of aflatoxins in food

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