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Review Article

A REVIEW ON BIOANALYTICAL METHOD DEVELOPMENT, VALIDATION AND TECHNIQUES USED FOR PHARMACOKINETIC STUDIES USING LC-MS/MS

Abdul Rahman MM, Kalyani Rupnawar, Madhuri A. Nagras, Supriya A. Unavane*
Department of Pharmaceutical Chemistry, STES's Sinhgad College of Pharmacy, Vadgaon (BK), Pune-411041, Maharashtra, India.

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ABSTRACT

Development and validation of bioanalytical method is important to understand the pharmacokinetics of any drug and/or its metabolites. Advanced techniques like liquid chromatography tandem mass spectrometry (LC-MS/MS) play very important role. Current advances in bioanalytical method development and validation is due to advancement of analytical instruments and extraction techniques. This is particularly true for chromatographs and detectors. This article reviews recent progresses and mathematical models which could assist in understanding and selecting adequate conditions to develop and validate selective and robust method, by utilizing hyphenated instruments like liquid chromatography tandem mass spectrometry. This article also reviews various extraction techniques like liquid–liquid extraction, solid phase extraction, protein precipitation, which play important role in sample preparation and detection by LC-MS/MS. The data obtained from these studies are used to support regulatory filings; it is directly related to bioanalytical studies. It also reviews some spotting methods like dry matrix spotting (DMS), dry blood spotting (DBS) and stripping method like charcoal stripping (for endogenous) substances.

1 INTRODUCTION

The trustworthiness of analytical method is prodigiously significant in analysis and is of course, essential for precise and accurate analysis of data (Imre et al, 2008). Bioanalytical field deals with analysis of drugs, their metabolites and/or endogenous substances in the biological matrix. A properly validated bioanalytical method for quantitative determination of drugs and their metabolites (analytes) plays quite important role in the effective performance of bioequivalence, pharmacokinetic (pk) and toxicokinetic studies. The data obtained from these studies are used to support regulatory filings. Validation ascertains the excellence of analyst's work. Users of bioanalytical data get assurance of the performance of method. Also, validation is mandatory by the regulatory agencies (Imre et al, 2008). In many cases, highly specific mass spectrometric detection, particularly by the use of tandem mass spectrometry (MS–MS) needs least separation on column. It will shorten assay period and make it possible to analyze many analytes within short frame of time (Ganesan et al, 2010). MS/MS provides high degree of selectivity. It can also analyze many samples in short period of time by multiple reactions monitoring mode (Zhang et al, 2000). Liquid chromatography tandem mass spectrometry (LC-MS-MS), because of

* Corresponding author. Tel: +91 9766900980; e-mail: supriya.unavane@yahoo.com
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its high degree of sensitivity, selectivity and robustness, has come out as a great tool in pharmaceutical industry for many analytical needs. Current article reviews vital information in bioanalytical method development including an efficient sample preparation (Kamalraj et al, 2012) and also shows various spotting techniques like dry blood spotting (DBS), dry matrix spotting (DMS) and stripping technique like charcoal stripping (for endogenous substances).

2 METHOD DEVELOPMENT

A well-organized development and validation of analytical methods are important components in the development of pharmaceuticals (Breaux et al, 2003). The legitimate experimental techniques that result from these developmental procedures are utilized by quality control laboratories to guarantee the identity, purity, potency and bioavailability of drug products (Breaux et al, 2003; Singh and Shah, 2011). A well-developed method should be easy to validate. A method has to be developed with the objective to be able to instantly check clinical samples, formulation prototypes, and commercial samples. By advances in method development and validation procedures, the evidence congregated is taken into consideration for the design and proceeding for development of method. Preferably, the validation protocol must pursue a complete understanding of the method's performance and intended use. The validation protocol will list acceptance considerations that this method may come across (Breaux et al, 2003; Findlay and Khan, 2010). A suitable protocol should be made for method development and validation technique. To create a relevant bioanalytical method, the experts should initially understand the rationale of trial so as to understand which analytical information is going to be employed, prerequisite of the method, and outcome of the results after complete conclusion of investigation. As soon as these aspects are well-defined, a suitable method can be established (Kamalraj et al, 2012). Method development consists of three essential inter-related parts: sample preparation, chromatographic separation and MS detection (Chandramouli et al, 2010).

2.1 Instrumentation

LC-MS/MS method associates the physical separation performance of liquid chromatography with mass analysis capabilities of mass spectrometry (Kamalraj et al, 2012). Recently, liquid chromatography tandem mass spectrometry (LC-MS/MS) has appeared as the supreme approach and authoritative method for quantifying drugs and their metabolites in biological matrix because of its great sensitivity, specificity, rapid rate of analysis and quick data compilation (Savoie et al, 2010).

A full-scan LC/MS examination can suggest probable oxidative and/or conjugative metabolic conversion by considering ionic species observed. Tandem mass spectrometry (MS/MS) is the practice of carrying out one mass selective operation after another. The purpose of first ionization is to separate an ion species called as parent ion or molecular ion, while that of latter ionization is to find out the mass to charge ratio (m/e) of the fragment or daughter ion, produced due to collision induced dissociation (CID) of the parent ions (Link, 2011).

The instrument can operate either in the full scan mode or in the more sensitive selected-ion monitoring mode (SIM) detecting positive or negative ions. For MS/MS analysis four different operating combinations are possible: Parent-ion scanning (scan mode in the first analyser, SIM in the second analyser), daughter-ion scanning (DIS; SIM in the first analyser, scan mode in the second analyser), constant neutral-loss scanning (CNLS; scan mode in both analysers) or selected-reaction monitoring (SRM; SIM in both analysers). SRM is also called as multiple reactions monitoring MRM. DIS is preferable for the identification of drugs and or their metabolites in complex matrices. Separation is performed on the LC and in the first mass analyser, while structural information is obtained by fragmentation in the second analyser (Link, 2011).

2.2 Sample Preparation

Sample preparation is very important step for analysis of drugs and metabolites in bioanalytical study.

Biological samples contain various complexes like proteins, endogenous and exogenous substances, together called as "unwanted substances" (Kamalraj et al, 2012). These can interfere with the drug in plasma which is present in very small quantities like picogram/ml to nanogram/ml range. The "unwanted substances" can interfere with small concentrations of the drug during analysis. The main objective of sample preparation is to eliminate all possible undesirable substances without significant loss of analyte of interest (Kamalraj et al, 2012). Currently several techniques are available for extraction of analyte from the biological matrices like; solid phase extraction (SPE), liquid-liquid extraction (LLE) and protein precipitation (PP).

2.2.1 Solid Phase Extraction (SPE)

The principle of SPE is based on the partitioning of the analytes between two phases. The analyte of interest must have higher affinity to solid phase than the matrix components. Now-a-days, SPE sorbent is packed between two fritted disks in the cartridge made up of polypropylene. The liquid phase is thus allowed to pass through the cartridge. Silica-base with chemically bonded functional groups or highly cross-linked polymers such as styrene divinylbenzene and polymethacrylate are generally used materials for preparation of solid phase cartridges. These cartridges can be utilized only once so as to avoid any interference and mostly to eliminate carry-over effects. Different types of SPE sorbents used are non-polar, polar and ion exchange sorbents. For reverse phase SPE, the stationary phase is usually silica bonded with alkyl and/aryl functional groups. C18 and C8 are the most common sorbents used. The packing materials required for SPE and liquid chromatography are comparable; only particle size is more in SPE as compared to that of liquid chromatography. SPE technique is modified by solid phase microextraction (Said, 2010; Gad, 2008).

Advantages

- Low concentration of drug can be detected.
- Effective in selective removal of interferences.
- Different types of adsorbents can be used.
- Extending the analytical column life, reduced system maintenance, minimizing ion suppression.

Limitations

- Extraction is difficult for high-density materials.
- In extraction processes a number of steps are to be carried out making it a time consuming process.

2.2.2 Liquid-Liquid Extraction (LLE)

The principle of liquid-liquid extraction is the distribution of analyte between two immiscible liquids (aqueous and organic). Extraction of analyte occurs from aqueous phase into organic phase. After extraction of analyte the organic layer is separated from aqueous phase and it is evaporated in presence of nitrogen gas so as to get the dry form of sample. Now-a-days traditional LLE has been replaced with advanced and improved techniques like liquid phase microextraction (LPME), single drop-liquid phase micro extraction (DLPME) and supported membrane extraction (SME) (Said, 2010).

Advantages

- Clean extracts obtained.
- Greater sample capacity.
- The technique is simple, rapid and has relatively less cost per sample.

Limitations

- Sometimes pH control of the sample necessary for extraction.
- During evaporation, since the temperature is increased, the method cannot be used for thermolabile substances.

2.2.3 Protein Precipitation (PP)

Protein precipitation can be applied to extraction of plasma and blood samples. Principle of PP is based on precipitation (denaturation) of the proteins by using various reagents like acid (trichloroacetic acid and perchloric acid), organic solvents (methanol, acetone and acetonitrile) or by salts (ammonium sulphate). After denaturation the sample is centrifuged, which results in extraction of analyte in the precipitating solvent. Methanol is generally preferred solvent amongst the organic solvent as it can produce clear supernatant which is

appropriate for direct injection into LC-MS/MS. Salts are other alternatives to acid and organic solvent precipitation. This technique is called as salt-induced precipitation. As the salt concentration of a solution is increased, proteins aggregate and precipitate from the solution (Said, 2010; Gad 2008).

Advantages

- This method can be applied for extraction of both hydrophobic and hydrophilic substances.

Limitations

- Protein precipitation may clog the column.

3 METHOD VALIDATION

The guidelines for bioanalytical method validation are published by the United States Food and Drug Administration (USFDA) in May 2001. These guidelines are standard for validation parameters' evaluation and requirements (Chandramouli et al, 2010). Bioanalytical method validation is the approach employed to indicate that the analytical method used to assess an analyte in biological matrix is reliable and also reproducible (Savoie et al, 2009). There are three types of validations; full validation, partial validation, and cross validation.

3.1 Full Validation

When carrying out bioanalytical method development of an analyte for the first time, full method validation is employed. Full method validation is mandatory for any new drug entity. Also full method validation is recommended when metabolites are added to current assay of already existing analyte. The assay is required to be revised for all analytes measured (Shah et al, 2000; Shah, 2007).

3.2 Partial Validation

Partial validations are usually modifications of validated bioanalytical methods which do not essentially require complete revalidations. In partial validation either one intra-assay of precision and accuracy is carried out or "approximately" full validation is done. Distinctive bioanalytical method variations can be included in this class, but are not restricted to bioanalytical method changes involving laboratories and analysts, instrument and/or software applications and platform steps. Partial validation can also be carried out when there is alteration in species within matrix (e.g. rat plasma to mouse plasma), changes in matrix within a species (e.g., human plasma to human urine), change in analytical methodology (e.g., change in detection systems), change in sample processing procedure(s), change in anticoagulant in harvesting biological fluid (Shah et al, 2000; Shah, 2007).

3.3 Cross Validation

Cross validation is comparison of two bioanalytical methods. Cross validations are essential when two or more bioanalytical methods are applied to generate information within same study. The evaluations should be done by considering an innovative validated bioanalytical method as the reference and the repeated bioanalytical method as the comparator and vice-versa. Cross validation with spiked matrix and subject samples should be carried out at each site or laboratory to create inter-laboratory reliability when sample analyses within a single study are carried out at more than one site, or more than one laboratory. This should be taken into consideration when data is generated by using various analytical techniques (Shah et al, 2000; Shah, 2007).

Method validation is done by the use of specific validation parameters which explains that the performance characteristics of the method are appropriate and reliable for the proposed analytical applications. The vital parameters for validation include: carry over effect, selectivity, sensitivity, dilution integrity, matrix effect, accuracy & precision, stability, recovery, purity of working standard, and exchange reaction. The suitability of analytical data relates directly to the criteria used to validate the method (Shah et al, 2000; Shah, 2007).

3.4 Carry Over Effect

Carry over effect in the assay is used to study the rinsing cycles or wash program of auto sampler required to wash the injection needle properly so as not to get any interference from the previous injection. For carry over recovery comparison

sample of 1.5 to 1.8 times of non-extracted standard H (highest calibration curve standard) containing internal standard, extracted sample containing 1.5 to 1.8 times concentration of standard H with internal standard, LLOQ sample in duplicate with internal standard from biological matrix and extracted blank samples from the same matrix lot are used (Hughes et al, 2007).

3.5 Selectivity

Selectivity exercise is carried out to assess the ability of the bioanalytical method to differentiate and quantify the analyte(s) in presence of other components in the sample. For selectivity, analyses of blank samples of appropriate biological matrix (plasma, urine, or other matrix) obtained from atleast six sources should be carried out. Each blank sample should be tested for interference, and selectivity should be ensured at the lower limit of quantification (LLOQ).

3.6 Sensitivity

Sensitivity is measured in terms of LLOQ (lower limit of quantification). For sensitivity six matrix spiked LLOQ samples using the same spiking dilutions should be used and the same for the preparation of LLOQ standard calibration curve (Kamalraj et al, 2012).

3.7 Dilution Integrity

This exercise is carried out to assess the ability of the method to quantify the analyte and yield accurate and precise results after the sample dilution. Dilution integrity test can be used when the subject sample concentrations are above the validated calibration curve range. For dilution integrity six sets of quality control samples are prepared by diluting them twice and four times prior to extraction by addition of screened interference free blank matrix (Kamalraj et al, 2012).

3.8 Matrix Effect

Matrix effect is investigated to ensure that selectivity and precision are not compromised within the matrix screened. Three blank samples from each of at least six batches of matrix under screening are extracted. For matrix effect LQC (lower quality control), MQC (middle quality control) and HQC (higher quality control) spiking dilutions and internal standard dilution are spiked in the above extracted blank samples. Recovery comparison sample at LQC, MQC and HQC concentration level along with internal standard are prepared and screened (Cappiello et al, 2008; Chiu et al, 2010; Patel, 2011).

3.9 Accuracy & Precision

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. Precision is reported in terms of coefficient of variance (% CV) over the range of quantitation for a single experiment. In this parameter standards are assayed in replicate (Intraday) and for a series of experiments in which standards are assayed over several experiments (Interday) (Kamalraj et al, 2012).

3.10 Stability

Drug stability in a biological fluid is a function of the storage conditions, the chemical properties of the drug, 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 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 (incurred sample reanalysis). 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 and interference free biological matrix. Stock solutions of the analyte for stability evaluation should be prepared in an appropriate solvent at known concentrations (Nowatzke and Woolf, 2007).

3.11 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 un-extracted standards that represent 100% recovery (Kamalraj et al, 2012).

3.12 Purity of Working Standard

Purity of working standard exercise is performed to evaluate the interference due to internal standard (deuteriated) at retention time or m/e ratio of analyte. For evaluating purity of working standard, recovery comparison samples containing internal standard are prepared and injected in triplicate. Also recovery comparison samples of lower limit of quantitation (LLOQ) concentration of analyte are prepared (in absence of internal standard) and injected in triplicate.

3.13 Exchange Reaction

Exchange reaction exercise is performed to evaluate conversion of deuteriated internal standard into analyte during sample preparation. For exchange reaction plasma samples containing an anticoagulant are spiked with internal standard, processed and injected in triplicate. Also analyte containing lower limit of quantitation (LLOQ) (in absence of internal standard) is prepared in plasma, processed and injected in triplicate.

4 SPOTTING AND STRIPPING TECHNIQUES USED IN PHARMACOKINETIC STUDIES

4.1 Dry Blood Spotting (DBS)

Application of whole blood on filter paper is called dry blood spotting (Li et al, 2011a; 2011b; Findlay and Khan, 2011). Currently DBS is actually employed for therapeutic drug monitoring, toxicokinetic and pharmacokinetic studies (Hannam et al, 2010; Li and Tse, 2010). It utilizes needles for pricking the finger; thereby collection of blood sample causes minimum pain (Turpin et al, 2010).

Advantages

- Lesser sample required which reduces the number of animals used.
- Very small amount of blood is sampled at each time point than plasma or serum analysis and other biological samples (Turpin et al, 2010).
- Invasive sampling method, offer easy sample collection.
- Storage over sufficient period without any deep freeze and expensive instruments and easier transfer (Li and Tse, 2010).

Limitations

- In spite of gaining high popularity of DBS-LC-MS/MS, the technique has its boundaries in sensitivity because of very small sample size (Li and Tse, 2010).

4.1.1 Dry Blood Spot Sample Collection

Volatile, air sensitive and/or unstable analytes are not suitable for spotting on DBS card/paper. Samples of DBS are collected by piercing finger, heel or toe with a lancet. The blood drops produced by pricking are usually spotted on to preprinted circles on DBS card/paper. Precaution must be taken that only blood spot should touch the card, if not it will cause spreading of spot or may produce contamination. After blood is spot, it is required to be set aside for few hours (mainly 2 hours) so that it will result in complete drying of spot. The sample collection volume should be very small between 15- 40µl, to obtain good results. Sample volume above 40µl causes saturation and the results obtained may not be appropriate. The detailed procedure of blood collection, transfer of blood on card/paper and transportation of sample is described by National Committee for Clinical Laboratory standards (Li et al, 2011a; 2011b; Li and Tse, 2010).

4.1.2 DBS Paper/Card

Whatmann papers which do not contain any additives and are made up of only pure cotton

linters are available. They are approved by FDA as a class-2 medical device. The paper/card shows good analyte holding ability. Besides this pore size and thickness are required to be taken into account as they play significant role and affect loading capacity and spreading ability of blood. Uniformity and absorption properties of various lots of papers must be similar. Cellulose is used for preparation of Whatmann paper/cards which gives strength to the paper (Li and Tse, 2010).

4.2 Dry Matrix Spotting (DMS)

The analysis of fluids other than whole blood is known as dry matrix spotting (DMS). DMS method can be used as an alternative to traditional techniques of analysis for extraction and detection of low volume assays or matrices like plasma or urine. These matrices need storage of samples in deep freezers (Christianson et al, 2007). Dry matrix spotting technique is a new and developing method in field of bioanalytical research. This technique is able to maintain analytical accuracy of the method. It also permits investigators and researchers to significantly reduce expenses and time duration required for analytical steps associated with liquid and solid phase extraction techniques. Main applications of DMS include drug metabolism and pharmacokinetic studies. Bond Elute DMS sample preparation card is generally used for dried matrix spotting. The size of blood spot depends on hematocrit level (percentage of blood made up of red blood cells) which fluctuates from person to person (Christianson et al, 2007).

Advantages

- It provides consistent spot shape, size and reproducibility, regardless of ratio of red blood cells to blood sample volume.
- In dry matrix spotting the spot having matrix, generally plasma, does not contain whole blood and thus spot variability in spots and drug concentration is generally not seen in dry matrix spotting as compared to dry blood spotting.

Limitations

- This technique also shows challenge in sensitivity because of very small sample size.

4.2.1 Dried Matrix Spot Sample Collection

Dried Matrix Spot (DMS) sampling is a method of collecting biological matrices obtained from human or animal subject and spotting it on DMS card/paper. For detection of spotted portion, disc shaped area of every spot is punched out from DMS card/paper. The punched circular discs are then extracted by using different techniques suitable for analysis. The extracts obtained are analysed by high sensitivity LC-MS/MS instruments.

Advantages

- Small sampling volume and easy sample handling.
- The samples collected by DMS technique are easy to store and does not require deep freezer. Thus, it provides significant cost saving.

Limitations

- It can pose limitations in sensitivity due to small sample size.

4.2.2 Paper/Card

Bond Elute DMS is a non-cellulose card/paper and is not soaked into reagents. Due to absence of impregnation this avoids non-specific binding of analyte of interest. This results in increasing mass spectrometry response and also improves the signal-to-noise ratio. Bond Elute DMS is compatible with standard punching tools and automation, and it needs five times less punching force as compared to cellulose based cards. This causes faster workflow, less technician fatigue and smoother automation.

4.3 Charcoal Stripping

Charcoal stripping (adsorption) is a process used to remove hormones and steroids (endogenous substances) from serum or plasma, required for bioanalytical study. It has also been utilized for improving immunoassay systems. The active binding sites on the charcoal bind with hormones or steroids, charcoal is removed by centrifugation. Filtration will efficiently eradicate remainder charcoal in serum or plasma (Mittelstaedt, 2007). Charcoal: dextran stripping decreases the serum concentration of many hormones and certain growth factors, such as estradiol, cortisol, corticosterone, vitamin B, T_3, T_4 and

prostaglandins. This serum is useful where these endogenous molecules may interfere with experimental work. It is made by exposing the serum to a mixture of dextran coated activated charcoal for a certain period of time (Anonymous, 2012).

5 CONCLUSION

Pharmaceutical companies are taking efforts to search out several new methods and technologies with regard to proficient drug development and manufacturing. These efforts are carried out to improve human health with minimum unwanted effects. To encounter these challenges employment of advanced analytical methods and high throughput automated platforms are used now-a-days. This can accomplish more research investigations in smaller duration of time and with improved data quality and reliability. Explanation of complete analytical technique under different circumstances can be accomplished accurately through ideal model preparation. These performances might be reflected as summary of outcomes of the completed study trials. More over method development can be implemented with additional organized and focused way so that models can be utilized with regard to future trouble-shooting purpose. A variety of practical study trials rely extremely on the amount of ideal detailed information and excellence of developed method. An outline of suitable validation method is offered to encourage concepts and the method to follow when suitable method is developed. An effort has been made to recognize and also describe bioanalytical method validation from fundamental point of view. On the other hand bioanalytical method development and its validation mainly rely on the experience and the preference of the developer. Current improvements in method development and validation are a result of advancement of detectors and chromatographs. Also few methods like dry blood spotting (DBS), dry matrix spotting (DMS) and charcoal stripping shows some advances in bioanalytical field for sample analysis. Dry blood spot and dry matrix spot can be used as an alternative to present method. These methods require very small sample size and have simple collection method with minimum pain to patients. These methods are also economical and space saving; as these methods avoid the use of deep freezer for storage of collected blood and plasma samples. These methods are also time saving and provide ease in shipment of sample. Charcoal stripping play important role in detection of endogenous substances and accurate results can be obtained.

CONFLICT OF INTEREST

There is no conflict of interest amongst the authors.

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