**BIOANALYSIS AND PHARMACOKINETICS: AN UNDERSTANDING THROUGH LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY**

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**Abstract**

Bioanalytical method development is the process of creating a method to identify and measure a chemical of interest in a biological matrix*.* A substance may frequently be measured using a variety of techniques, and selecting an analytical technique requires careful thought. To evaluate drugs and their metabolites in biological matrixes, a variety of extraction procedures, including protein precipitation, liquid-liquid extraction (LLE), and solid phase extraction (SPE), are extraction techniques for sample preparation.Drugs and their metabolites are quantitatively analyzed using bioanalytical procedures in biological media including saliva, urine, plasma, and serum. The Pharmacokinetic characterization required for lead compound selection frequently becomes a rate-limiting step in the process of developing a drug. Samples from these extraction techniques are spiked with calibration standards (CS) and quality control (QC) samples. The analytical method development process involves sampling, sample preparation, chromatographic separation, detection, and result interpretation. Evaluation and interpretation of bioavailability, bioequivalence, pharmacokinetic, and pharmacodynamic investigations heavily rely on bioanalytical validations. where several parameters are measured, including accuracy, precision, selectivity, sensitivity, repeatability, and stability. The process by which it is determined that the performance characteristics of a bioanalytical technique satisfy the needs of the intended bioanalytical application is known as validation. Full validation, partial validation, and cross-validation are the further divisions of the validation, each of which serves a different function.

**Introduction**

A new drug discovery can cost near or more than one billion dollars, and it can take ten to twelve years for the treatment to be commercially available. The process of creating drugs and analyzing each one's attributes to determine whether it is possible to choose one new chemical entity (NCE) and turn it into a safe and effective form of drug is known as drug discovery and development. Pharmacokinetics (PK) and Toxicokinetic play an important role to drug discovery and development with LC-MS/MS technique. A sensitive and focused bioanalytical approach is crucial given the emphasis on PK/Toxicokinetic and the higher potencies of newer drugs. It is widely acknowledged and known how important a tool bioanalysis has become in the field of drug discovery and development. A large number of assays, including those for important metabolites, have been continuously developed for NCEs (New Chemical Entity) to support various stages of research and development with the use of the LC-MS/MS technology for detection and quantification purposes. Bioanalytical techniques are used for the identification and quantification of generic and prescription drugs. Clinical studies can be greatly benefited from the bioanalytical data produced in discovery and pre-clinical programs. These programs data on plasma concentration-response can be compared to human results and these comparisons are especially helpful.

**PHARMACOKINETICS:**

Understanding pharmacokinetics involves studying how a drug moves through the body and gathering information on its overall exposure over time. This quantitative analysis looks at how the drug moves through different systems in the body such as the brain, plasma, blood, kidney and other organs to track its concentration levels at different time intervals. We study three key pharmacokinetic processes absorption distribution metabolism and excretion. By understanding a drug's pharmacokinetic profile we can develop the best dosing schedule to optimize therapeutic benefits and minimize side effects.

**Absorption:**

Drug absorption occurs when it moves from the administration site to the systemic circulation, which provides a functional blood supply to all tissues. The extent and rate of absorption are determined using bioavailability, which refers to the fraction of the drug that is present at the site of action. For example, in the case of oral route administration, the drug typically passes through the small intestine and also the liver, where a portion of it is eliminated or metabolized before entering the bloodstream. As a result, there is a decrease in the drug amount delivered to the site of action, resulting in reduced bioavailability. Intravenously administered drugs exhibit 100% bioavailability because they directly enter the bloodstream, so the absorption is not primarily measured in this case in the first place. Food can sometimes affect drug pharmacokinetics, especially with oral dosage forms, and can provide data on drug absorption based on the impact of high and low-fat meals, and fasting states. Gastric acid-reducing compounds may also be studied to determine if a clinically important drug-drug interaction (DDI) should be expected as a result of simultaneously administered medicines that elevate gastric pH. The factors which influence absorption are:

* The chemical characteristics of the drug,
* Drug formulation,
* Route of administration and site of administration,
* and interactions with other drugs and food.
* Patient physiological state

**Distribution:**

Distribution takes place next after absorption. The drug is subsequently transported all over the body when it is absorbed. The process of a drug travelling to various organs, tissues, and regions of the body is known as distribution, and it relies on a number of variables, such as the drug's chemical makeup, fluid condition and blood flow. The volume of distribution, a significant PK parameter, quantifies distribution. The volume of distribution, which reveals how much of the drug is in the tissues as opposed to the blood, is essential for figuring out the dosing schedule and half-life. Protein binding is another crucial factor that needs to be considered while researching the distribution mechanism. One such plasma protein that a drug may bind to when it enters the bloodstream is albumin which is the most available plasma protein in our body. Protein binding causes the drug to stay inactive as it is bound to the protein. To get pharmacologically active this drug needs to be free from protein binding. Even in getting metabolized, the drug needs to remain unbound. To know more about this property of the drug several protein-binding assays (PPB) are done in the lab.

**Metabolism:**

The process of metabolism happens following absorption and distribution all over the body. The process of making chemical alterations of a drug molecules to produce new compounds that may serve as the drug, producing pharmacological effects, or that may aid in excretion is metabolism or biotransformation. Metabolites are substances that are generated as a result of metabolism. The GI tract, plasma and kidneys, are among the sites of the body where metabolism may take place, although the liver is where this happens most often. Different enzymes that are found inside the liver can metabolize medicines through Phase I and Phase II metabolic pathways. In general, pharmacologically active metabolites are produced during phase I reactions. The nature of the drug is typically altered during phase II reactions, making a molecule more likely to be water soluble and thus pharmacologically inactive. Despite the fact that drug metabolism decreases its pharmacologic activity, prodrugs are drugs that become active after metabolism. Prodrugs are frequently created with specific parent drug qualities so that they exhibit improved ADME characteristics and increase the medicine's effectiveness for the treatment of the patient. The majority of medications are processed by CYP enzymes, or cytochrome P450. The body's ability to eliminate pharmaceuticals is aided by CYP-mediated drug biotransformation, which also has a major effect on the action and safety of medications. Drug-Drug Interactions can result from simultaneous or combination drugs exerting an adverse effect on CYPs, which contribute to alterations in drug responsiveness. The following variables impact drug metabolism:

* Genetic Factors
* Drug interactions
* Age
* Organ failure (e.g renal/hepatic)

**Excretion:**

Drugs that are not removed by metabolism are removed by excretion. Although there are many different ways that drugs can be eliminated, including through the liver, skin, lungs and GI tract, the kidneys are by far the most prevalent pathway. A drug's pharmacokinetics may be impacted due to impaired renal function when the kidneys are the primary route of excretion. Reduced elimination of the drug or its metabolite is one form of alteration in the PK of the drug that may result from this. Renal impairment can prevent the medicine or its metabolites from being effectively eliminated, which can lead to drug buildup in the body and can lead to potential toxicity. Therefore, it's critical to understand the subject's physiology before beginning the PK investigations in many programmes to ensure that the correct dose can be delivered. In particular, for medications with short therapeutic indices, adjusting dosage regimens in patients having renal impairment can assist minimise buildup at steady-state, reducing side effects in that population. The following variables may have an impact on medication excretion:

* Medical issues that affect the renal flow
* Intrinsic drug characteristics, including size and pH.
* Genetic diversity
* Age.

**BIOANALYSIS**

Bioanalysis is a method used to determine the concentration of drugs, metabolites and endogenous substances in biological matrices such as plasma, blood, urine, etc. Bioanalytical methods are often used for studies in pre-clinical studies, toxicology, and clinical pharmacology to measure drugs and their metabolites. The bioanalytical approach utilized for the quantitative evaluation of NCEs and Standard drugs in biological fluids evaluates and interprets bioequivalence, pharmacokinetics, and toxicokinetic research.It aids in conducting clinical research as well as pharmacodynamic, toxicological, pharmacokinetic, bioequivalence, and therapeutic drug monitoring (TDM) studies. In their early phases, this research is only carried out in toxicological studies and to determine overdose situations. Pharmacokinetic parameters are computed and generated from the drug concentration in the biological matrix. Studies on bioanalysis are crucial for developing new chemical entity such as drugs.

Bioanalysis is a method for analysis a drug's therapeutic efficacy. Therefore, bioanalysis is significant in the pharmaceutical sector. In the bioanalysis process, there are three main processes including sample collection, sample preparation and analyte detection and quantification techniques.

* **ASSESSMENT OF DRUGS IN VARIOUS BIOLOGICAL MEDIA**

For biopharmaceutical investigation, samples of blood, urine, and faces are frequently taken, especially if the drug or metabolite is poorly absorbed or significantly excreted in the bile. Other media, such as saliva and tissue, may also be used for analysis. The choice of the sample media is greatly influenced by bioanalytical technique. Clinical pharmacokinetic research requires the use of blood, urine, and saliva to measure drug levels. Drug levels in blood and/or urine may be needed for bioavailability research, although a drug identification may just need one type of biological sample to be resolved.

The matrix frequently makes drug or its metabolite detection in biological medium more difficult. For this problem, different sample clean-up techniques are used such as protein precipitation and liquid liquid extraction, to successfully separate desired components from endogenous biological elements. The effectiveness of the clean-up technique set a limit on the test method's sensitivity and selectivity.

When blood is centrifuged after clotting, maximum 50 percent of the blood's original volume is extracted as serum. The increased production of plasma from blood makes it commonly chosen matrix for analysis. In bioanalytical research Protein denaturation procedures may be necessary before further operations are carried out on blood, serum, or plasma.

After collecting blood, it should be quickly centrifuged at 4000 to 5000 rpm for around 5 to 10 minutes and the supernatant (serum or plasma) should then be transferred using an appropriate tool, such as a pipette, to a clean container. The patient's urine is one of the biological matrixes which is easy to collect samples that are often larger in volume. There is no need for denaturation processes since there is no protein in the urine of the person. They are widely examined in drug metabolism since they are the main source of metabolites studies.

* **PROCESSING AND STORAGE OF BIOLOGICAL SAMPLES**

In a bioanalytical investigation, sample preparation is a crucial step in the examination of drugs and metabolites. Proteins and other endogenous and exogenous substances that might affect the analyte are present in biological samples. The goal of sample preparation is to remove all potentially undesirable components from the analyte of interest while minimizing analyte loss. Therefore, while choosing the material of containers for sample storage and preservation, attention should be made otherwise it can interfere with certain drug analysis.

* **DRUGS ESTIMATION IN BIOLOGICAL FLUIDS**

The choice of sample medium is greatly influenced by the kind of drug study. In a clinical pharmacokinetic investigation, the drug levels must be determined using biological samples. Drug levels in blood or urine may need to be assessed for a bioavailability study. The estimate approach of pharmaceuticals in biological fluid consists of many phases, including sample collection, sample preparation, separation of the component of interest from the matrix, and analysis.

* **BIOLOGICAL SAMPLE EXTRACTION PROCEDURE**

Sample preparation is the process of cleaning up a sample before analysis and/or concentrating it to improve detection of analyte of interest from biological fluids like plasma. After sample collection and before sample (analyte) detection and quantitation, this technique is known as "bioanalytical sample preparation".

Preparing a bioanalytical sample has the following goals:

1. Elimination of undesired matrix elements, primarily proteins, that would affect the detection of analytes.

2. Increasing the analyte concentration to the analytical instrument's detection threshold.

3. Changing the analyte's solvent or solution to make it compatible with mobile phase before injecting it into a chromatographic device.

4. Diluting a solvent to weaken it or make it compatible with another solvent.

5. Analyte stabilization to prevent enzymatic or hydrolytic breakdown.

Once the appropriate analyte has been selected, it should be removed from the biological fluid with extraction procedure. Since there are several extraction strategies that may be used to prepare samples, this step in the bioanalytical process is very important. The sample preparation process is time-consuming and should be done properly because it is one of the crucial steps for bioanalysis. Liquid-liquid extraction is used to reduce matrix effects and when the sample is in liquid form such as whole blood, plasma, and urine. If the biological matrix is solid, liquid-solid extraction is employed.

Some of the various extraction methods include dilution followed by injection, solid phase extraction, protein precipitation, filtration, liquid-liquid extraction, equilibrium dialysis, ultrafiltration, restricted access media, solid-supported liquid-liquid extraction, monolithic columns, and immunoaffinity extraction. Some of the various extraction methods include solid phase extraction, protein precipitation, dilution followed by injection, filtration, ultrafiltration, liquid-liquid extraction, equilibrium dialysis, solid-supported liquid-liquid extraction, and immunoaffinity extraction.

The most well-known and often employed extraction procedures among all of them are: Liquid-liquid extraction (LLE), solid-phase extraction (SPE), and protein precipitation techniques.

* **Protein precipitation technique**

The interaction of the precipitation reagent with protein groups provides the basis for protein precipitation. In general, soluble proteins contain a hydrophobic core encircled by a hydrophilic surface made up of ionic groups unrelated to intramolecular interaction. Protein intra-molecular hydrophobic interactions are disrupted by organic solvents.

The serum proteins precipitate when a volume of a solvent (often acetonitrile/methanol) is added, which result the analyte moves from the matrix to the solvent. This analyte can then be directly injected or sometimes before injection it can be dried down and reconstituted in a lower volume to concentrate the analyte. Although this method of sample preparation is the quickest and easiest, but it can result in ion suppression or enhancement problems, particularly in ESI method, where the coelution of endogenous substances other than proteins such as phospholipids, and fatty acids impact the ESI droplet desolvation process.

* **Liquid-Liquid Extraction**

The method known as "liquid-liquid extraction" is frequently used to separate analytes from liquid matrices. Divide or distribute the sample between two immiscible liquids or phases to isolate the analyte from interferences. The two phases of LLE are typically an organic solvent (generally the lighter phase) and an aqueous solution (often the denser or heavier phase). While hydrophobic molecules will choose the organic solvent, hydrophilic compounds will favor the polar aqueous phase. By dividing the sample between these two immiscible liquids or phases, it is incredibly effective for isolating analytes from interferences.

The fundamental idea is that analytes have a strong affinity towards one solvent over another immiscible solvent. The approach is based on the Nernst distribution law, which states that any species will disperse between two immiscible liquids so that the ratio of the concentrations remains constant. The ratio of the concentration in octanol to the concentration in water is known as Log P, is the often-cited partitioning coefficient of a species.

Two immiscible solvents are introduced to the biological fluid and centrifuged before the organic solvent is removed. More surface area is needed for quick equilibrium, which can be produced by vigorously mixing or hand-shaking. Aqueous form of extracted analytes can be placed straight into HPLC, the residue so obtained is reconstituted with the appropriate small volume solvent compatible with HPLC separation. This technique allows for the extraction of several samples. Several actions must be taken in order to use this strategy efficiently. They are:

* A solvent with a low boiling point is required to make the extraction process easier.
* To make mixing with the sample easier, a low viscosity solvent is required.
* The analyte has to be soluble in the extracting solvent.
* Low solubility in water, high purity, and compatibility with HPLC detection methods are all requirements for the organic solvent to be chosen.
* **Solid Phase Extraction**

In the sample preparation method known as solid-phase extraction (SPE), based on their physical and chemical properties, substances that are suspended or dissolved in a liquid mixture are distinguished from other compounds in the mixture. Solid phase extraction may be used to separate desired analytes from a variety of biological matrices. Using the affinity of solutes dissolved or suspended in a liquid for a solid through which the sample is passed, SPE separates a mixture into desired and undesirable components. As a consequence, either the sample's undesirable contaminants or the desired analytes of interest are kept on the stationary phase. The portion that travels through the stationary phase is either collected or discarded depending on whether it contains the analytes or the contaminants. If the portion that is still on the stationary phase contains the necessary analytes, they can then be removed from it for collection in a following step in which the stationary phase is washed with the appropriate eluent.

The solid and liquid phases are both present for the solid-phase extraction technique. In this procedure, the analyte is held on the solid phase as the sample moves through, and it is then eluted using a suitable solvent. Here, a plastic disposable column or cartridge is employed as the solid phase. It is packed with C-18 silica, a reversed phase substance and a hydrocarbon phase. SPE technique includes the selective transfer between a liquid and solid phase to separate the analytes from the biological sample in a solid-liquid phase.

Depending on the needs of the experiment, these sorbents, which can be polar, non-polar, or ionic in nature. Similar to liquid-liquid extraction (LLE), the fundamental idea behind SPE is the partitioning of solutes across two phases. Instead of two immiscible liquid phases, like in LLE, SPE involves partitioning between a liquid (sample matrix or solvent containing analytes) and a solid (sorbent). This sample treatment method enables the concentration and purification of analytes from solutions as well as the purification of extract after extraction by adsorption on a solid sorbent. The typical procedure is to load a solution onto the SPE solid phase, filter out the undesired components, and then extract the desired analytes into a collecting tube using a separate solvent. In solid phase extraction, the packing is first conditioned before the sample is added, the packing is then washed, and finally the sample is eluted.

* **Analytical techniques for quantifying pharmaceuticals in body fluids**

For the quantitative detection of drugs in body fluids, a variety of techniques are used. These techniques are chosen based on the biological fluid that need to be quantitated. Radioimmunoassay (RIA) methods, enzyme-linked immunosorbent assay (ELISA), capillary electrophoresis, chromatographic techniques such as high-performance liquid chromatography (HPLC) with UV/PDA type detector, liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), and gas chromatography (GC) are all examples of analytical techniques.

* **The use of chromatography in bioanalytical studies:**

For drug research and clinical trials, liquid chromatography with a UV or fluorescence detector has been utilized effectively. Compounds with low level dosages, such inhaled products, need the development of methods with the requisite sensitivity, which might demand a certain amount of laboratory time and derivatization protocols.

Shorter run times in chromatographic procedures can be achieved without the necessity for entire analyte resolution by using selective ion monitoring (SIM) and multiple reaction monitoring (MRM).

Chromatography technique with a detector platform may be used to separate, detect and measure the concentration of a drug and its metabolites with bioanalysis in biological fluids, which is helpful for preclinical, clinical, and drug discovery and development processes. For bioanalytical research, hyphenated methods like LC-MS and GC-MS are critical and crucial. Chromatography makes it possible to separate certain compounds and determine their quantities with the help of detecting instruments such as UV, PDA and Mass Spectrometry. In chromatography, the separation of chemicals is based on many theories.

* **Internal standard**

By adding a predetermined quantity of a compound to a specified amount of sample to create separate peaks, this method makes up for the chemical of interest being lost during sample preparation. It is usual practice to utilize the quantitation technique with the inclusion of an internal standard to correct different analytical mistakes. The substance used as the internal standard should not interact with the sample in any way and should exist fully independently of it. If there is a chance that the substance will be lost during processing, an internal standard is added to the material to be analyzed in chromatographic analysis.

In this manner, regardless of the volume of solution lost, the internal standard and sample ratio concentration stay constant. Any reduction in compound interest will also result in a reduction in internal standard by an equivalent fraction. The internal standard chosen should have qualities similar to those of the compound of interest, however any other compound with different properties may also be used. It is common practice in chromatographic analysis to use internal standards.

Internal standard should maintain the following criteria:

* It should elute in close proximity to the analyte of interest, with no interferences visible in the peak.
* It must be stable and doesn't react with the target molecule, the mobile phase, or the column materials.
* It should behave like analogous to the molecule under study, such as pretreatment, derivatization, etc.
* It should be provided in extremely pure form.
* It should be added at a concentration that results in a peak area that is equivalent to that of the analyte of interest.
* **Method Validation in Bioanalysis**

Drugs and/or their metabolites may be evaluated and identified in biological fluids or tissues using analytical methods in clinical and forensic toxicology, or they may be quantified in these matrices, or both with different bioanalytical techniques. An analytical procedure is considered validated if its suitability for the intended analytical application has been established by laboratory testing. Any new or updated technique has to be validated to ensure that it can deliver consistent and dependable results when used by different operators using the same tools in the same or other labs.



* **Specificity**

The capacity to clearly evaluate the analyte in the presence of components that could be anticipated to be present is known as specificity. These often include things like contaminants, degradants, matrix, etc. Other supporting analytical procedures may make up for a single analytical procedure's lack of specificity.

* **Accuracy**

A measured value's accuracy refers to how closely it matches the real or accepted value. The difference between the mean value discovered and the true value is accuracy. For the determination purpose applying the technique to samples that have known quantities of analyte added. To make sure there is no interference, these should be compared to both standard and blank solutions.

Acceptance criteria: Accuracy within 15% of nominal value (20% near LLOQ).

* **Precision**

The degree of closeness between a set of measurements, which is achieved by serial sampling of the same homogeneous sample under the specified conditions, is expressed by the precision of an analytical technique. There are three types of precision: repeatability, intermediate precision, and reproducibility.

Acceptance criteria: Precision within 15% R.S.D. (20% near LLOQ).

* **Limit of Detection (LOD)**

The smallest amount of an analyte that chromatographic separation can detect; nevertheless, it's not required for this amount to quantify as an exact number. Signal to noise ratio must be calculated for both blank and analyte with blank and analyte chromatograms. The concentration is then determined when the signal to noise ratio is about 3:1.

LOD = 3.3\*SD/S.

where SD is the response's standard deviation and S is the calibration curve's slope.

Acceptance criteria: Compliance with identification criteria or Signal to Noise ratio value ≥ 3

* **Limits of Quantitation (LOQ)**

It can be identified by the smallest amount of an analyte that can be accurately and precisely measured.

LOQ = 10\*SD/S.

Where S is the slope of the calibration curve and SD is the standard deviation of the response.

Acceptance criteria: Compliance with accuracy and precision criteria near LLOQ or Signal to Noise ratio value ≥ 10

* **Linearity and Range**

The correlation between the response and the analyte's known concentration is called a calibration curve. Every analyte should have its own calibration curve, which should be created in the same biological matrix as the samples. The concentration range (interval between the upper and lower concentrations of the analyte) for which the technique has been verified in terms of accuracy, precision, and linearity is known as the range. The most basic model that accurately captures the concentration-response relationship should be utilized as the calibration curve.

* **System Suitability**

A system suitability test is performed to determine if the chromatographic as well as mass spectrometric system's sensitivity, resolution, and repeatability are suitable for the intended purpose of the analysis. The most important variables in determining a system's applicability are the tailing factor, the retention period, the resolution, etc.

* **Robustness**

An analytical procedure's robustness is measured by its ability to be unaffected by small, purposeful changes to the technique parameters, and it gives a indication as to its dependability under typical conditions.

* **Solution Stability**

The stability of standards and samples is determined during validation under typical conditions, under regular storage conditions, and occasionally under specific storage conditions, such as refrigeration or protection from light, are required.

**Matrix effect (ion suppression/enhancement)**

In LC-MS/MS analysis, the phenomena of analyte ionization suppression or enhancement by coeluting chemicals is well recognized, and it mostly depends on the sample matrix, sample preparation technique, chromatographic separation quality, mobile phase additives, and ionization type. While ESI has been said to be far more prone to these effects, atmospheric pressure chemical ionization (APCI) has less contribution for these effects. Ion suppression and enhancement both obviously have the potential to influence validation parameters including linearity, accuracy, and precision in the absence of an internal standard that has been isotopically tagged or analogous form of analyte. Therefore, ion suppression/enhancement investigations have to be a crucial parameter of any LC-MS/MS method's validation.

* **Conclusion:**

The first step towards more intelligent drug development is to build your programme around a thorough knowledge of pharmacokinetics. For the FDA and other regulatory authorities to approve a medicine's safety, effectiveness, and other aspects relating to patient usage, a good understanding of the ADME characteristics and PK of a therapeutic molecule is essential. We may infer from this chapter that appropriate bioanalysis is necessary in order to understand the drug's pharmacokinetic characteristics. Different extraction techniques, rules, and method development are key elements that affect the molecule's bioanalysis and meet accurate pharmacokinetic data for the same.

* **References:**

1. Tripathi, K. D. *Essentials of medical pharmacology*. JP Medical Ltd, 2013.
2. Rang, Humphrey P., Maureen M. Dale, James M. Ritter, Rod J. Flower, and Graeme Henderson. *Rang & Dale's pharmacology*. Elsevier Health Sciences, 2011.
3. Eyer, Charles L. "Goodman & Gilman's: The Pharmacological Basis of Therapeutics." *American Journal of Pharmaceutical Education* 66, no. 1 (2002): 95.
4. Sunil, S., BREEN JAMBHEKAR, and J. PHILIP. *BASIC PHARMACOKINETICS*. Pharmaceutical Press, 2022.
5. Pushpa Latha, E., and B. Sailaja. "Bioanalytical method development and validation by HPLC: a review." J Appl Pharm 1 (2014): 1-9.
6. Kirthi, A., R. Shanmugam, M. Shanti Prathyusha, and D. Jamal Basha. "A review on bioanalytical method development and validation by RP-HPLC." Journal of global trends in pharmaceutical sciences 5, no. 4 (2014): 2265-2271.
7. Snyder, Lloyd R., Joseph J. Kirkland, and Joseph L. Glajch. Practical HPLC method development. John Wiley & Sons, 2012.
8. Chang, Min S., Qin Ji, Jun Zhang, and Tawakol A. El‐Shourbagy. "Historical review of sample preparation for chromatographic bioanalysis: pros and cons." Drug Development Research 68, no. 3 (2007): 107-133.
9. Munson, James W. "Pharmaceutical analysis: modern methods. B." Drugs and the pharmaceutical sciences 11 (1984).
10. Prabu, S. Lakshmana, and T. N. K. Suriyaprakash. Extraction of drug from the biological matrix: a review. IntechOpen, 2012.
11. Reddy, K. V., and Y. Yachawad. "Overview on recent extraction techniques in bioanalysis." International Research Journal of Pharmacy 7, no. 2 (2016): 15-24.
12. Wells, Martha JM. "Principles of extraction and the extraction of semivolatile organics from liquids." Sample preparation techniques in analytical chemistry 162 (2003): 37-138.
13. Burgess, Richard R. "Protein precipitation techniques." Methods in enzymology 463 (2009): 331-342.
14. Majors, Ronald. "Practical aspects of solvent extraction." The Application Notebook (2009).
15. Wells, Martha JM. "Handling large volume samples: applications of SPE to environmental matrices." Solid-Phase Extraction: Principles, Techniques, and Application, (2000).
16. Żwir-Ferenc, Agata, and Marek Biziuk. "Solid Phase Extraction Technique--Trends, Opportunities and Applications." Polish Journal of Environmental Studies 15, no. 5 (2006).
17. Ahuja, Satinder, and Stephen Scypinski, eds. Handbook of modern pharmaceutical analysis. Vol. 3. Academic press, 2001.
18. Pandey, Saurabh, Preeti Pandey, Gaurav Tiwari, and Ruchi Tiwari. "Bioanalysis in drug discovery and development." Pharmaceutical methods 1, no. 1 (2010): 14-24.
19. Peters, Frank T., Olaf H. Drummer, and Frank Musshoff. "Validation of new methods." Forensic science international 165, no. 2-3 (2007): 216-224.
20. Murugan, S., A. Elayaraja, K. Chandrakala, P. Ramaiah, and Chathurya Vulchi. "A Review On Method Development And Validation By Using HPLC." International journal of novel trends in pharmaceutical sciences 3, no. 4 (2013): 78-81.
21. LK, TIJARE, RANGARI NT, and MAHAJAN UN. "A review on bioanalytical method development and validation." Asian J Pharm Clin Res 9, no. 3 (2016): 6-10.
22. Bhardwaj, Santosh Kumar, K. Dwivedia, and D. D. Agarwala. "A review: HPLC method development and validation." International Journal of Analytical and Bioanalytical Chemistry 5, no. 4 (2015): 76-81.
23. Sharma, Shivani, Swapnil Goyal, and Kalindi Chauhan. "A review on analytical method development and validation." International Journal of Applied Pharmaceutics 10, no. 6 (2018): 8-15.