Review Article

A REVIEW ON BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR ESTIMATION OF ANTIVIRAL DRUGS BY USING HYPHENATED TECHNIQUES AND ITS APPLICATIONS ON ROUTINE ANALYSIS

ABSTRACT:

Bioanalytical methods are used to create a protocol that can identify and quantify bioactive molecules and their metabolites in human and animal samples. For determining the medication and its metabolites present in human/animal body, the bioanalytical method is efficient. One of the prominent bioanalytical roles is creating new techniques, validating existing processes, and analyzing samples. Most importantly, a compound can be calculated using many methods and can also be detected using various analytical methods. In specific biological samples, the drugs can be evaluated by different extraction procedures, such as solid-phase extraction, liquid extraction, protein precipitation etc.., All the steps in methods were investigated to know how environment, sample, or techniques influence the matrix assessment up to the study's time. The analytical methods, including high-pressure (HPLC), liquid chromatography combined with dual-mass spectroscopy (LCMS-MS), and Ultra-performance extraction (UPLC), can be used to obtain the in-depth analysis of medications. All have their strengths and weaknesses. Currently, bioanalysis is usually done by HPLC-MS and GCMS-MS. Linearity, repeatability, Accuracy, precision, selectivity, recovery and consistency are essential criteria. In this review we proposed to give importance of bioanalytical system production and validation parameters to aid in drug quality, purity, safety and efficacy.

KEYWORDS: Bioanalytical, extraction procedures, UPLC,LC-MS/MS.

INTRODUCTION

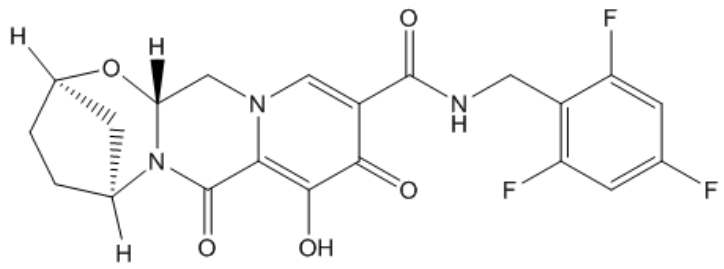
The adoption of the bioanalytical techniques is among the major drug development bottlenecks. Bioanalytical technique validation is employed to determine the various analytes in biological media accurately. Current bioanalysis heavily relies on properly hyphenated devices and processing conditions. During the drug design or development process, biomedical research businesses must develop comprehensive bioanalytical procedures (1)

Identification and estimation of tissue samples are included in bioanalysis (blood, serum, plasma, skin, saliva, hair, urine, feces, and various organ tissues). Smaller compounds such as medicines and respective metabolites are detected in bioanalysis, while proteins, peptides and other larger entities are recognized. As a result, bioanalysis is a crucial tool in numerous scientific domains, including drug development, forensic investigation, toxicology, doping, and locating disease-specific biomarkers.

Bioanalysis is complicated because of the complex sample matrix [2–11]. Before using complicated samples in analytical equipment, there must be extensive sample processing. High-performance sample processing and conjugated analytical tools are necessary for advanced bioanalysis. The drug has been studied using liquid chromatography (LC) coupled with dual Mass Spectroscopy (MS/MS) for a long time and currently in Bioanalysis.

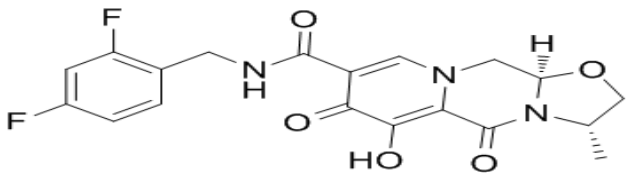
Bictegravir is a new HIV-1 integrase strand transfer inhibitor (INSTI) agent. HIV-1 multiplication is prevented and viral genome incorporation into the host organism is blocked. (12). It is manufactured by Gilead Sciences and has been approved by FDA in 2018. In comparison to previously approved INSTIs, bictegravir has excellent resistance and efficacy (13). Early antiretroviral treatment containing two nucleoside RTIs is advised to be given combined with INSTIs. (14). In b oth T-cell lines as well as human T cells, bictegravir demonstrate robust and specific antiretroviral action, with 50% effective concentrations and selectivity. The clinical trials analyse that Bictegravir-contains regimen had similar viral suppression and was not dissimilar to the DTG strategy (15-16). When combined with pther antivirals such as emtricitabine, or darunavir, bictegravir exhibits similar antiviral activity experimentally. It also demonstrates significant antiviral efficacy against HIV-1 strains that are tolerant to other antivirals (17). Bictegravir has rapid Pharmacokinetic activity as well as a half-life that is favorable for regular medication in HIV-affected persons (18). Overall study report states that Bictegravir appears to be extremely efficient Integrase transfer inhibitor as against other Antiviral drugs (19). Bictegravir has a better in vitro resistance profile compared to other INSTIs.(20).

Figure : 1 Chemical structure of Bictegravir



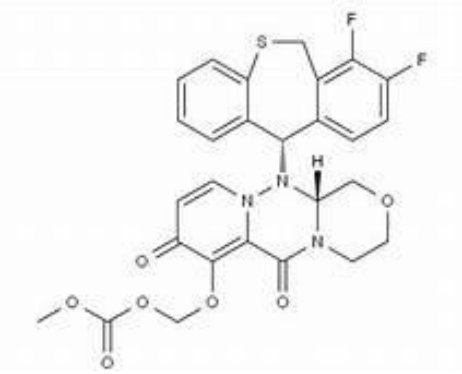
Cabotegravir is a potent HIV integrase strand transfer inhibitor(21) Because of the drug's prolonged plasma half-life, long-acting injectables that are given monthly(22,23) or once in two months were designed (24).` Pre-exposure therapy is another usage for this medication besides HIV therapy. In clinical studies, CBG with rilpivirine intramuscular administration showed pharmacokinetic diversity for both prophylaxis and therapy (25—27) There is a high chance of interactions with other medications, (28) such as long acting Injectables and AntiRetroviral drugs, and at there isn't enough clear evidence on how important they are clinically, and monitoring of Antiretrovirals plasma levels when new co medications at risk. Although multiple investigations (29) have established its circulating levels, no article has been written specifically about the development and validation of LC-MS/MS techniques.

Figure :2 Chemical structure of Bictegravir Cabotegravir



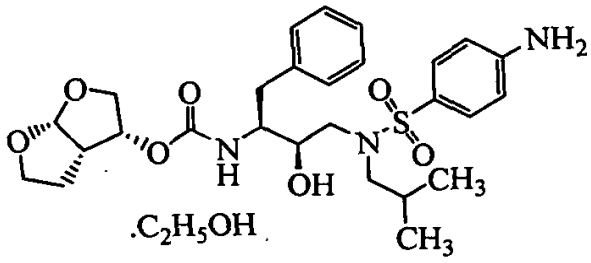
Baloxavir marboxil (BMX) is a specific Cap- Dependent Endonuclease antagonist that has been licensed for combatting influenza A and B virus infections. In humans, its prodrug form is converted to baloxavir acid, which prevents influenza A and B viral multiplication both in vivo and experimental models (30-35) In clinical studies, a single dose of BMX limited viral replication and faster effect in declining of influenza symptoms in patients(36). The FDA authorized BMX for therapeutic application in 2018. However, baloxavir acid exhibits strong and wide-spectrum suppressive activities towards seasonal, bird, and swine viruses in experimental studies. It has stronger inhibitory property towards type A virus as against type B (37).

Figure :3 Chemical structure of Baloxavir marboxil



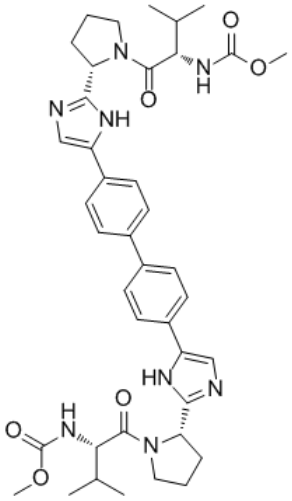
Darunavir is an efficient antiviral agent prescribed for managing HIV. It mechanism of action is inhibition of protease enzyme of HIV and inhibits breaking down of gag and gag polyproteins in viral afflicted cells. Darunavir has a greater affinity for and a greater adherence to the HIV proteolytic enzymes due to strongly binding of oxygen atoms with amino acids via hydrogen bonds.than with the other protease inhibitors,due to this Doravirine displays its excellent antiviral effectiveness targeting HIV variants (38). By preventing viral development and CD4+ T-cell invasion, this activity helps to limit the spread of other infections. Darunavir showes strong anti-HIV activity with lower cytotoxic effect [39]. In T-cells, the effectiveness of Darunavir is greater compared to other such drugs [40],due to formation of a highly stable complex between Darunavir and the protease enzyme for its flexibility and backbone interactions [41]. Numerous studies provide evidence of the substantial restriction to HIV resistance to darunavir. [42-44].

Figure :4 Chemical structure of Darunavir



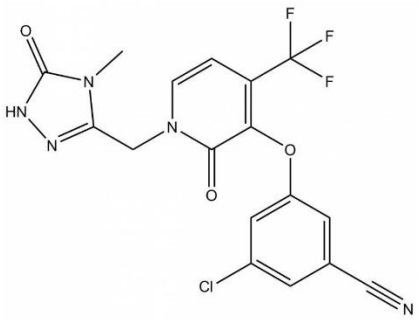
Daclatasavir is a medication that fights the hepatitis C virus directly and is prescribed for managing persistent genotypic infections of that virus (45). The endogenous materials can be found in the blood systems of several animals. In serum/plasma, daclatasavir has a strong affinity towards hydrophilic molecules, and its higher extraction recovery (ER) contain sources, which is a new method for GLP.(46) So as such cases, it is need to develop faster development techniques with protein precipitation , solid phase extraction (SPE) , and liquid–liquid extraction (LLE) used to extract with eco-friendly solvents resolves its complex nature with the effect of analytical and biological matrices.(47).

Figure :5 Chemical structure of Daclatasavir



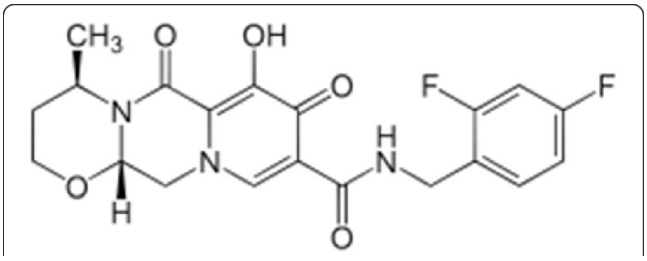
Doravirine (MK-1439) is a newly licensed NNRTI for managing HIV-1 infected individuals in combination with other antiretroviral drugs [48]. At present, the other NNRTIs that have been licensed for clinical therapy which includes efavirenz (EFV), nevirapine, etravirine and rilpivirine, exhibits limited genetic obstacle to tolerance and significant cross-resistance [49]. A strong genetic barrier limits the risk of developing tolerance to doravirine, which has substantial efficacy against other HIV variants [50]. In comparison to ritonavir and EFV, phase III trials illustrated non-inferior effectiveness with a better neuropsychological characteristics and more satisfactory lipid levels [51]. According to US Department of Health and Human Services standards, Doravirine is useful for people who developed resistance to EFV as it has a variety of adverse effects, particularly mental and neuronal problems that prompted EFV to be downgrade and going for an alternative therapy. [52].

Figure : 6 Chemical structure of Doravirine



Dolutegravir is a HIV integrase antagonist that is typically administered in conjunction with other antiretroviral medications for managing HIV infection (53). Dolutegravir does not cause rapid liver damage and is related to a minimal effect on raised levels of serum aminotransferase during therapy. Dolutegravir is a carboxylic acid amide made through classical condensing of the carboxylic group with amino group of 2,4-difluorobenzylamine.(55). It is an organic tricyclic molecule, a secondary carboxamide and a difluorobenzene. It is a conjugate acid of a dolutegravir(1-).(56)

Figure : 7 Chemical structure of Dolutegravir



SAMPLE PREPARATION

The sample preparation for research is frequently the most significant and challenging step, both with respect to time and the uncertainty of extracting the required analyte out of matrix. Furthermore, every matrix faces a different set of challenges. While plasma includes a lot of phospholipids, urine has a significant salt content. RBCs, lysed blood cells, as well as other components make up whole blood. There are frequently differences between every analyte and matrix, which determine the appropriate extraction process (57).

THE COMPOUND DETECTION

An MS detector is the mpst appropriate one. (58) The European Commission action use HPLC-MS/MS with both ESI and atmospheric pressure chemical ionization (APCI) technologies are the principal techniques used in quantitative biological research (59). Packaging material of 1.7 μm particles has been available through recent technological progress. Ultra high-pressure liquid chromatography (UPLC), coupled with a high-pressure pump and a high-speed Mass Spectrometer, provides a rare high-performance and greater resolution for maximum chromatographic efficiency. (60)

OBJECTIVES OF SAMPLE PREPARATION (61)

Lessen the effects of the matrix

To separate sample for variability test

Decrease assay uncertainty

More sensitivity

Samples are more pure

UNDER STANDING THE DIFFICULTIES IN METHOD DEVELOPMENT (62)

The matrix

The quantity of samples

Urgent approach

The frequency of analyses that were quantified

Pharmaceutical description of analyte

BLOOD: PLASMA RATIO

The blood: plasma (Kb/p) ratio is the concentration of drug in blood (CB) to its concentration in plasma (CP), in whole blood containing RBCs, plasma etc..,. (63)

FINANCIAL ASPECTS OF DRUG DEVELOPMENT OF LARGER MOLECULE

Approximately more than ten years and about nearly $1 billion is required to discover and design a novel drug compound. There are about nine large compounds approved each year, compared to 23 per year for small molecules. Therefore, it is essential at the beginning of drug production to assess the candidates objectively. The drug failure at various phases during the manufacture and the patent expiration period,

particularly for already-available organic compounds, resulted in a decline in pharmaceutical R&D since Biologic is projected to achieve a market value of approximately $54 billion off-patent in the coming five years. The pharmaceutical industry is making significant efforts to reduce internal resources, expenses, and bioanalytical research. The need to make bioanalytic sound test help as a key tool for drug discovery and production must be well known, increasing the list of substantial pharmaceuticals expenditure. (64)

The various phases of drug research and analysis are widely acknowledged as being essential to the pharmacokinetic/pharmacodynamic characterisation of the new compound since it was discovered that led to its market authorization. There are a few general ideas in this field that are the foundation of an overarching structure for approaching Bioanalysis from the outset and through different phases of drug production (65)

EXTRACTION METHODS

LIQUID –LIQOID EXTRACTION

Principle: "It is based on selective extraction by the organic immiscible solvent of the intended analyte present in the liquid sample."

Dichloromethane , Ethyl acetate , Diethyl ether, Texans, etc., and Methyl her-Butyl Ether . Any solvent can be used as an extractive solvent as single or in conjuction with another apt solvent.

Liquid Liquid Extraction builds on different solubilities and divided into one of two immiscible fluids. In most cases, the two phases must be immiscible, one aqueous phase and one non-aqueous phase. The drug compound may be preferentially isolated from the matrix by selecting the suitable extraction technique and sample buffer if required.(66)

SOLID PHASE EXTRACTION (SPE)

It is centered on partition of solid phase (adsorbent) of an analyte by delay with a solid substance under a particular circumstances. Solid Phase Extraction is depend on mechanism for selective adsorption. When the target analyte is adhered onto the solid stage, it could be extracted by employing a suitable solvent for elution (66). In solid-phase extraction, various cartridges are used.

SPE steps: - Step 1. Conditioning: Suitable solvents are needed for each Solid Phase Extraction tube to be packaged before using the sample.

Methanol, small amounts of Dichloro Methane, and Tertiary butyl methyl ether are solvents and any other solvents like organic, water, and buffers can be used.

Step 2. Sampling Application: Applying the sample from the cartridge’s top slowly ensuring that no sample on the cartridge's inside wall drop without any braking is allowed. The slow speed is needed for retaining of analytes due to a short and temporary bond.

Step3. Rinder or Wash: Matrix components and other interferences can be removed by rinsing the cartridge using weak dilute solvent or buffer and weakly retained interferences that are drained out of cartridge by analyst. Air buffers of various pH are rinsing or washing solvents.

Step 4: Drying, the vacuum pump can be used for the recommended time of drying by applying a suitable vacuum. Drying time is recommended for 2-3 minutes.

Step 5. Elution: It requires the transmission of a solvent at slow rate through a cartridge,so that the total extraction efficiency can be packed more time. Methanol, Acetonitrile, alkaline or acidic Methanol or Acetonitrile elution solvents or any organic solvent mixed with Methanol and Acetonitrile or any appropriate combination are the small amounts of Di chloro methane, tertiary butyl methyl ether, and some other organic solvent. It is used to break weak bonds between analyte and sorbent and improve the elution of multiple small volumes (66).

1. **Reversed-phase SPE**

Reverse-phase separation involves a polar (mobile) and a non-polar (stationary) phase. The analyte is mid-polar to nonpolar. Several Solid Phase Extraction products, such as silica-containing alkyl and aryl (LC-18, ENVI-18). LC- 18(octadecyl bonded silicone) for nonpolar to polar reverse phase extraction of antibiotics, barbiturates, benzodiazepines, caffeine, dyes, essential oils, vitamins, phenol phenols (67).

2. **Normal phase SPE**

These techniques generally include a polar stationary Phase, a mild to nonpolar mobile phase includes acetone, hexane, and chlorinated solvents. under normal phase condition usually uses polar-functionalized bonded silica (e.g., LC-CN, LC-NH2, and LC-Diol) and polar adsorption mediums (LC-Si, SLC-Alumina). (67)

3. **Ion-exchange spe**

Exchange of Ions between compound and charge in a solution, Solid Phase Extraction can be used. LC-SAX or LC-NH2 bonded silica cartridges can be used with anionic compounds. The LC-SCX or the LC-WCX bonded cartridges are employed for different cationic compounds.

Benefits of SEP over LLE41 (68)

1. Comfortable way, 2. Ease of application. 3. Recovery and precision increased

4. Easier automation, 5. The total analyte fraction is more available.

PROTEIN PRECIPITATION

Principle: 'Solubility of analytes, specifically solvents found in the biological matrix, such as blood, serum, would depend on that.'43

Solvent: methanol, Acetonitrile, and so on.(69)

It denatures the proteins essentially. It may be performed with any of the following techniques:

The change in pH of the sample, and addition of organic solvents. In a another example, the hydrophobic surface of the protein leads to its denaturation, etc. Recent advances are techniques for sample preparation.

1. salting-out assisted Liquid liquid Extraction[salle]

2. Protein precipitation plates

3. solid-phase extraction technique

4. Salting-out assisted Liquid liquid Extraction

When a mixture of water and an organic water mixer has inorganic salt, then the solvent is separated from the mix. It is a biphasic system and is widely known as "separation of the salt-induced process." Not only is the salting method useful for separating water-mixable organic solvents, but also for extracting nonpolar organic solvents. Salt -out assisted Assisted LLE process used in the treatment of magnesium, ammonium sulfate, NaCl and CaCl. The SALLE process is economical and could be automated (70).

PROTEIN PRECIPITATION PLATES

Protein Precipitation plates (PPP) were designed for filtration in same well, with no centrifugation and supernatant transfer steps following protein precipitation. They have membrane filter tubes structurally and can be fastened to vacuum filtering. For easy performance of overall operation, the filtering plate is compatible with modern auto samplers.

Acetonitrile showes more leakage than methanol among the Protein precipitation solvents tested. The selection of PPPs for an analysis should includes precipitating solvents, filters, pores, vacuum pressure, non-specific analysis to the plates, and matrix effects (ion removal).

The conclusion is that Protein precipitation -plates give many advantages, (70):- compared to manual PP.

Centrifugation or transmission filtrate steps are necessary Lessened time to process Increased solvent recuperation and utility with inadequate sample volume.

Solid-phase extraction (SPE) technique

SPE works on the similar concept as liquid chromatography (71). SPE are: - Recently

Molecularly imprinted polymer SPE

Dispersive SPE

Disposable pipette extraction

Micro-extraction by packed sorbent

Solid-phase microextraction

Stir bar sorptive extraction

Online solid-phase extraction

The theory is that around a template, a polymer network, i.e., a print molecule, is formed (70), Forming the pre-polymerization intricate in-between the template and functional monomers. Molecularly -Imprinted SPE (72) Polymer

Molecularly imprinted polymer SPE applications: - Environmental samples may be evaluated

Food samples may be assessed

Veterinary samples may be tested

Dispersive solid-phase extract

In Dispersive Solid Phase Extraction the sampling solution requires extracting the solvent using organic solvents like acetonitrile-ethyl acetone, $MgSO_4$ separation, or in conjunction with another salt (like NaCl) and clean-up with a Dispersive SPE.

The dSPE method has traditionally been employed very frequently in (70):

Sample tests of pesticides

Environmental samples may be evaluated

Food samples may be assessed

Analysis of cosmetic additives

Common dSPE applications, 70

Antihelmine medicinal residues determination in milk.

Trace b removal

Pig tissue agonists

B-lactam antibiotic determination for the kidney tissue

Disposable pipette extraction

It is a modified standard pipette tip (1/5 ml) that can be dispersed free of charge and filled with free flowing sorbent powder. Using a regular tip, it is simple to take the solvent in and out of this scattering absorbent.

The typical shape includes a dispersible sorbent freely sandwiched between the frits one fridge positioned at tip's lower end, from where the solvent may be picked and the other fridge positioned at the top to prevent contamination by solvents in the pipette (70).

Micro-extraction by packed sorbent (MEPS)

These are in the shape of a syringes packed with 1 mg sorbent, (100-250 mL), and connected as a cartridge between the needle and barrel. The MEPS protocol involves pre-treating of dilution and centrifugation of complex sample. In case of plasma/serum and blood samples with proportions of 1:4 and 1:20, correspondingly, dilution accompanied by centrifugation is recommended. The sample can be drawn directly by a MEPS syringe. As the sample passes through the MEPS cartridge the analysts are tied to the sorbent. To improve the sensitivity of procedure the sample may be extracted and expelled multiple times using the same vial if the sample is necessary before the concentration. In the next stage, the sorbent bed is washed. The last stage is to elude analytes with an organic solvents of 20-50 mL. This stage may be performed directly in the equipment's injector (GC or LC) and MEPS may be added to plasma, hair, serum, saliva, urine, blood in bio-medical applications.

Solid-phase micro-extraction (SPME)

A modern technique is known as SPME using a fused silica fiber covered outside with a sufficient stationary stage. Physically, the syringe is adjusted and incorporates micro tubing of stainless steel within the needle. 1 cm fused silica fiber edge is used to polymerize the micro tubes. The plunger of the syringe, coated silica fiber can be move back and forth. Solid Phase Micro Extraction assembly design allows all sample preparation steps to be combined in one stage, minimizing specimen time, organic solvents, and disposal costs. There are two types of mining methods for Solid Phase Micro Extraction the first is the direct immersion (DI) of SPME, the fiber into sample matrix of liquid samples, called direct immersion (DI) of SPME, and the second is the extraction of the headspace (HS) in a vial, which is heated to dehydrate the analytes which is held in the fibers. Typically a process is called a SPME.

Stir bar sorptive extraction (SBSE)

The Stir Bar consists of a glass jacket magnetic rod, which is covered of certain thickness. As the sample is mixed , analytes divide the sample matrix into the extraction step. The overall fibre coated PDMS volume in Stir bar sorptive extraction is 0.5 ml ,The film is 100 mm thick. Higher extraction levels are anticipated to provide higher extraction yield compared to SPM in SBSE. SBSE has two extraction modes, including SPME, i.e., direct dipping and headspace (HS - there is the same system available to keep the stir bar.

Online solid-phase extraction (OLSPE)

There are two kinds of OLSPE columns, they are restricted access [RAM] column and the turbulent flow chronograph (TFC) column. RAMs are employed to analyze low molecular mass substances containing high molecular materials in complex matrices (e.g., medicines, endogenic compounds, and xenobiotics) (commonly proteins). RAM HPLC columns extract the sample clean-up and used for directing biological

samples, Hydrophilic/hydrophobic, ion-exchange or size exclusion mechanism, and also used in multi-component analyses of antidepressant medicines on biological fluids. (73).

Measurement

A specific analyte calculation may also be split into a separate process and detection process.

Separation

LC is a method of splitting a complex sample into a column and distributed it between two phases. The phase in the column is stationary, and the phase that passes through the column is mobile solvent. In this TLC, HPLC is often used.

table 1 : Detection by UV visible spectrometry drug in biological liquids Spectrometric fluorescence (74)

S.No	Title	MATRIX	COLOUMN	SAMPLE PREPARATION	Mobile Phase	Sample Injection	Flow Rate	REFERENCE
1	Development and validation of a multiplex UHPLC-MS/MS assay with stable isotopic internal standards for the monitoring of the plasma concentrations of the antiretroviral drugs bictegravir, cabotegravir, doravirine, and rilpivirine in people living with HIV	Human Plasma	Kinetex EVO C18 column	Protein Precipitation	Water,Formic acid and acetonitrile	50 µ L	300 µ L/min	75
2	Development and validation of LC-MS/MS method for quantification of bictegravir in human plasma and its application to an intracellular uptake study.	Human Plasma	Ultimate 3000 RS column compartment	Protein Precipitation	Water,Formic acid and acetonitrile	7 µ L	0.250 ml/min	76

3	Development and validation for high-performance mass spectrometry method for determination of baloxavir marboxil in biological matrices	Human Plasma	C 18 coloumn	Protein Precipitation	Formic Acid and Methanol	10 µ L	0.6 mL/min	77
4	Direct injection SLE-HILIC-MS/MS analysis of darunavir	Rat Plasma	Luna-HILIC (250 mm × 4.6 mm, 5 m) column.	Liquid liquid extraction	Water,Formic acid and acetonitrile		1 ml/min	78
5	A novel LC–ESI-MS method for the simultaneous determination of etravirine, darunavir and ritonavir in human blood plasma	Human Plasma	Agilent Zorbax® XDB C-8 (50 mm × 3.0 mm, 1.8 m) column	Liquid liquid extraction	Water,Formic acid and acetonitrile	1 µ L	0.75 ml/min	79
6	Validation of Simultaneous Quantitative Method of HIV Protease Inhibitors Atazanavir, Darunavir and Ritonavir in Human Plasma by UPLC-MS/MS	Human Plasma	Waters Acquity UPLC C18 (50 × 2.1 mm, 1.7 µm) column	Solid extraction	Ammonium formate, PH 4 and ACN	5 µ L	0.3 ml/min	80
7	Development and validation of LC-MS/MS method for simultaneous determination of sofosbuvir and daclatasvir in human Plasma: Application to pharmacokinetic study	Human Plasma	ZorbaxSB-C18 column (4.6 x 50 mm,5µm)	Liquid Liquid Extraction	Ammonium formate and acetonitrile	3 µ L	0.7 mL/min	81

8	Development of a Robust UPLC Method for Simultaneous Determination of a Novel Combination of Sofosbuvir and Daclatasvir in Human Plasma: Clinical Application to Therapeutic Drug Monitoring	Human Plasma	UPLC BEH C18 column	Solid Phase Extraction	Ammonium formate and acetonitrile	3 µ L	0.2 ml/min	82
9	Title: Efficient HPTLC-dual wavelength spectrodensitometric method for simultaneous determination of sofosbuvir and daclatasvir: Biological and pharmaceutical analysis	Human Plasma		Liquid liquid Extraction	Ethyl acetate and Isopropanol			83
10	Ionic liquid-based vortex-assisted DLLME followed by RP-LC-PDA method for bioassay of daclatasvir in rat serum: application to pharmacokinetics	Rat Plasma	Waters Xterra C18 column (250 × 4.6 mm; 5 µm)	Liquid Liquid Micro Extraction	ammonium acetate: ACN: MeOH (65:20:15, v/v/v).	20 µL,	1.0 ml/min	84
11	Determination of Doravirine in human plasma using liquid–liquid extraction and HPLC–MS/MS	Human Plasma	Reverse phase HPLC coloumn	Liquid liquid extraction	Water,Formic acid and acetonitrile	7 µ L	300 µ L/min	85
12	Development and validation of an	Human	Acquity UPLC BEH C-18	Protein Precipitation	Water and ACN with 1% FA	2 µ L	0.475 ml/min	86

	UPLC-MS/MS bioanalytical method for simultaneous quantification of the antiretroviral drugs dolutegravir, elvitegravir, raltegravir, nevirapine and etravirine in human plasma	Plasma	column, 1,7 µm, 2.1 x 50 mm (Waters, Etten Leur, The Netherlands)					
13	Validation and clinical application of a novel LC–MS method for quantification of dolutegravir in breast milk	Dried Breat Milk spots	reverse phase C18 Waters XBridge (3.5 µm: 2.1 × 50 mm) column	Liquid Liquid Extraction	of 0.1% FA in demineralized water or MeOH.	5 µl	5 µl/min.	87

ACN: Acetonitrile; **FA:** Formic acid; **MeOH:** Methanol

BIOANALYTICAL METHOD VALIDATION

Necessity for validation of the bioanalytical process

• It is crucial for well characterized and validated Bioanalytical techniques the accurate, consistent findings to be satisfactorily construed.

•Bioanalytical techniques are being changed and improved continuously.

Specific parameters for validation are necessary in each investigation and each Technique the specific characteristics differ from Analysis to analysis.

•The bioanalytical procedures should be verified at every site and across the different areas for inter laboratory consistency when analyzing samples for a specific research at many sites. (88)

LINEARITY

Linearity defines as the system's characteristic in which the test findings are directly proportionate to the sample analysis. Irrespective of the technique, the linear range of the procedure must be calculated. The top and the bottom levels assessed during the accuracy analysis shall be dependent on 5 concentration ranges. (89). Given below are the ICH guidelines for estimation of concentration ranges which are suggested to be considered for assessment in method validation:

•**Assay:** 80%–120% of concentrations of samples (finished product or pharmaceutical substances). Nevertheless, a detailed study must serve as the basis for this range. At the very least, the linearity must be raised to between 75% and 125%. At nominal values of 80, 100, and 120 percent, accuracy must be

established. Content consistency: Depending on the dosage form, 70 to 130% of the sample concentration are acceptable, except if a wider, highly suitable range is justifiable; (e.g., metered dose inhalers).

• **Method of dissolution:** ±20% of range is necessary., the linearity range should start to exceed 120 percent of the total medication content below the average quantity retrieved at the initial draw, if dissolution profiles are required

•**Method of impurity:** the degree of reporting to 120%

• **Combined form of impurity and testing:** The norm of 100% is used to quantify and 120% for disclose impurity.

Accuracy

An Accuracy defines the proximity of the procedure's results to the analyte's actual value. Replicated analyses calculate the Accuracy of samples that contain known research quantities.It should be determined by at least five concentration determinations—and at the very least 3 in the predicted range. The finding does not exceed 15 percent of the practical value, with the exception for LLOQ. The difference between the average and the practical reading acts as a precise indicator (90-91)

Bias

As per ISO, Bias is defined as discrepancy between expectations of test findings and agreed benchmarks .and involved two or more systemic error factor. Bias may be calculated as a divergence of percentage from the agreed standard value. The word trueness is the mean value of widely agreed reference value of a wide range of measures. It can be defined by way of partiality. The truthfulness is not generally calculated during process validation but during routine quantification from the findings of many QCs (92) due to the significant efforts required for analyzing such a comprehensive sequence.

Precision

Precision of the technique indicates the proximity of an analyst's measurements when repeatedly applied to several aliquots of a uniform biological array volume. Precision should be determined by utilizing a minimum of five concentration estimations. A minimum of 3 concentrations are needed in the range of estimated concentrations. The exactness of the variance (CV) coefficient calculated at every concentration shall not surpass 15% with the exception of LLOQ, where the concentration level shall not surpass 20% of CV. Precision may be additionally divided in to inter- day, intra-day, and various analysts. Precision or repeatability steps were taken that assess Accuracy across time and require various analysts, different instruments, reagents, and lab facilities (93).

Intermediate precision

Intermediate precision refers to changes: various days, observers, instrument, etc. (94) The ISO coined the phrase "intermediate precision M-factor different" when the M-factor reflects the number of variables, which vary in successive estimations (analyst, instrument or time). Intermediate precision is referred to as intermediate precision (95) or between days.

The Detection Limit (LOD)

The LOD is only a function of the limit test in which the modest quantity of analyte in a sample may be identified in the given experimental conditions but not quantified. The detection typically refers to %, components per million, or ppm etc..,

The Quantification Limit (LOQ)

The LOQ is define as the limited quantity of analysis in a sample that may be measured with acceptable precision and Accuracy. And it is the most practical approach which is possible to choose LLOQ according to precision and Accuracy. The LLOQ is the modest quantity of sample, which can be quantified accurately and precisely. For instance, only if baseline noise, chromatographic methods (96), is applied LLOQ depending on the signal and noise ratio.

Robustness

According to ICH recommendations, the capability of an analytical technique to sustain reliability over the course of routine application without being affected by minute alterations in process characteristics (97-99). The capability of reproducing the procedure in a different lab settings or under different conditions, which is an experimental setup to evaluate the robustness of a method, avoiding experiencing unequal changes in the obtained findings.

Ruggedness

The extent to which test findings achieved under typical test settings by examining the identical samples can be reproduced is the ruggedness of the analytical methodology (100) This includes different analysts, laboratories, columns, tools, reagent supplies, drugs, and mobile phase.

Recovery Study

The recovery should be consistent, accurate, and reproducible but should not be 100 percent. Recovery assays must be conducted by utilizing unextracted criteria, representing 100% of reconstruction, for extracted samples' analytics at 3 levels (lower, moderate, and higher). (101-103)

Matrix Effect

Matrix effect is define as the impact of biological sample's elution along with remaining matrix on target ionization the Organic and inorganic molecules, such as amines, and carbohydrates, etc., cause the Matrix impact. IS-normalised matrix factor CV determined from a six lot matrix does not exceed 15 percent . It may be achieved at lower and higher concentration level (maximum 3xLLOQ) and close to the upper LOQ. The estimated average CV does not surpass 15 percent for the concentration. (104-106)

Stability

Accurate determination throughout the analytical procedure depends on the analyte's stability, which is an essential prerequisite. Stability assays during the different research stages, such as pre-analysis preservation, are also necessary for the system to be fully validated. (107)

1**. Sustainable stability** in the same vessels, under the conditions of storage as anticipated with authentic samples at the same temperatures and over some time, the sample matrix's stable should be maintained.

2. **Thaw stability**: As samples, the analyte's stability should also be evaluated during various freezing/thaw cycles. The authors approved that at two concentrations, the conference's reporting needs a minimum of three periods.

3. **In -process stability** Stability of the analysis is tested under sample preparation conditions which includes ambient temperature overtime necessary to prepare the sample. It is commonly agreed that this kind of stability must be assessed during sample preparation to determine whether preservatives need to be included to avoid analyte (108-110)

SPECIFIC RECOMMENDATION FOR BIOANALYTICAL METHOD VALIDATION

A basic model is utilized for sufficient weighting and statistical tests on fitness explain the concentration-response relationship (111)

For Validation of Bioanalytical process, Precision and Accuracy should be defined by at least five determinations by concentration level (not including blank samples). With the exception of LLOQ, the mean does not deviate by above 20% from the conceptual interpretation by 15 percent. 20% of the CV, the Accuracy around the medium value shall not surpass 15% of the CV. Further techniques to evaluate precision and Accuracy that follow these limitations might be suitable as well. (112)

In case of Instrument Failure the Re-injection reproductivity need to be assessed to ascertain if an analysis run (113)

DOCUMENTATION FOR METHOD ESTABLISHMENT

• For the technique implementation and setup the operational definition of analytical technique should be included in the documentation.

• In validation studies the Proof of pure drug standards and identity, metabolite standards, and internal standards. (114).

• Stability studies definition and data support. The experiments were carried out to be accurate, correct, recover, selective, LOQ, calibration curve, and relevant outcomes obtained from those tests. (Equations and weighting functions, if any)

• Documentation of precision and Accuracy of intra- and inter-assays.

• Information concerning cross-validation findings, where appropriate, in NDA submissions.

• Legible or mass spectrograms annotated, if necessary and
• any deviance from GLPs (as appropriate) and explanations for such deviance, SOPs, procedures, or (Good Laboratory Practice). (115)

OTHER INFORMATION

The following details includes: lists of abbreviations, references along with their readable copy, and any other code used, such as the study's conditions, integration and reporting codes.

(116-118) the following areas include SOPs or protocols:

• Acceptance or rejection criteria standard calibration, • acceptance or rejection criteria curve calibration, • acceptance of QC samples and run time criteria or rejection criteria,

• Conditions of Acceptance for recorded findings during double-checking of unidentified samples;

• Classification of the code, such as sample codes for clinical or preclinical and bioassays, • Arrangement of clinical or preclinical lots for testing,

• Gathering, treatment, and preservation of samples and

• Repetitive sample analyses, sample reintegration.

APPLICATION OF VALIDATED TECHNIQUE FOR ROUTINE INVESTIGATION

For stability data the Assessments of all analyte samples in a biomatrix must occur in the available time. In general, validation data is a single finding with no doubling or replicating analysis may be used to test biological samples when the assay method has suitable variability. (119) this applies to processes that consistently fall within appropriate tolerances with Accuracy and precision variables.

Response function: The study's standard curve should use the same fitting, weighting, and fitness defined during pre-study validation. The response feature during validation decide by Proper statistical testing based on the actual standard points. There are possible issues with the changes in response functions' connection from pre-study validation to routine running validation.

• To approve or deny the sprint, QC samples should be utilized. The matrix of such QC samples is spiked by the analyte. (120)

System suitability: A particular SOP (or sample) should be selected based on the analysis and methodology to ensure optimal functioning of the system.0

Repeat analysis: an SOP or guide for acceptance requirements is essential which have a explanation of reason for Repeat analysis. The Reasons for repeated testing includes repeated assessment off clinical or preclinical samples' for regulatory reasons, non-consistent analysis of replicates, samples exceeding test

range, sample treatment inaccuracy, malfunctioning of equipment, weak chromatograms, and inconsistent PK results. If the sample volume permits, re assays should be performed three times and should be clearly known the rationale for repeat testing and disclosing the repeat study.

Reintegration of sample data: A SOP or a guideline should be provided for the sample data reintegration. And The motives for reintegration and procedure to carry out reintegration should also be outlined in this SOP or guideline. It is necessary to clearly explain and record the reason for reintegration. Reports should be made of actual and reintegration results.

Conclusion:

In this Article a complete focus on estimation of Antiviral Drugs in Biological Samples by Hyphenated techniques were different concentrations of drug in biofluids like Plasma, serum, saliva, tissues etc.., can be determined which leads to analysis of different applications such as Biological significance, Pharmacokinetic study, Bioavailability, Bioequivalence, Toxicity, Half life, Drug Discovery, Disease Biomarkers, forensic study etc.., can be done. This techniques is not only applicable to Antivirals but other available drugs by using its Active Pharmaceutical Ingredient. This review also focus on different Extraction techniques by which high content of extracts can be obtained . Different Analytical parameters can be calculated and validated which is used for Routine Analysis.

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