An Exploration of Advancement in Analytical Methodology for Quantification of Anticancer Drugs in Biomatrices

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Significant numbers of newer anticancer drugs are regularly entering into the market worldwide to fight against different types of cancers. Analytical methodologies are being developed to quantitate those molecules in a variety of matrices during their drug development stages. Selection of biological matrices for developing bioanalytical methods is based on the mechanism of action, site of action, site of metabolism and route of excretion of the drugs or their metabolites. In this review, we have described the current scenario and advancements in bioanalytical techniques for quantification of different anticancer drugs in a variety of biomatrices with a special emphasis on sample preparation techniques. We have discussed and summarized different bioanalytical aspects for anticancer drugs, which can give direction to the researcher for choosing appropriate techniques for their quantification needs.

Keywords Bioanalysis, sample preparation, biological matrices, anticancer drugs

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1 Introduction

Among the major treatments available for cancer, chemotherapy is one of the most preferred.†‡ Scientists are continuously engaged in research worldwide to discover effective and specific anticancer drugs having minimal toxicity. With an increasing number of anticancer drugs, it is of utmost importance to develop advanced analytical techniques for their quantification in biological samples during the drug discovery and development processes. Numerous detection methods are being reported for quantification of these agents in different biological matrices.

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Initially, liquid chromatography with an ultraviolet detector (LC-UV) was used for detection, but later mass spectrometry (MS) modernized the analysis. High-performance liquid chromatography (HPLC) still remains one of the most widely used analytical techniques. Other techniques include capillary electrophoresis with an ultraviolet detector (CE-UV), capillary electrophoresis with laser-induced fluorescence detector, gas chromatography-mass spectrometry (GC-MS), infrared spectroscopy, and Raman spectroscopy. Bioavailability is primarily evaluated based on the drug concentration present in the blood or plasma. Analysis of drugs in different matrices is required for monitoring the therapeutic level of a drug and fixing the individual dose for a patient. Sometimes, plasma concentration of a drug alone is not sufficient to reflect its efficacy or toxicity and hence, analysis of a drug in cells or tissues becomes necessary. Estimation of the drugs or their metabolites in intracellular compartment is important when the target receptors or metabolizing enzymes are present inside the cells. The anticancer drugs of nucleoside analogue class are one example of such type of drugs that require intracellular drug level analysis. For analysis when any agent is present in low amounts, then the concept of micro-dosing is used with the help of a sensitive detection technique. Dose individualization is important for the chemotherapeutic agents that have severe adverse effects. Biological matrices like plasma, urine, serum, cerebrospinal fluid (CSF), tissue, etc. exert their own criticality during sample preparation in bioanalysis.

The literature includes many reports on the analysis of individual anticancer drugs, either in a pharmaceutical formulation, environmental samples or biological sample. However, this is the first report on different analytical aspects and methodologies for the quantification of anticancer drugs covering almost all biological matrices. In this review, we have discussed the techniques of analysis of anticancer drugs published in the last 10 years and organized them based on different biological matrices. We have summarized the sample preparation techniques and chromatographic conditions of each individual drug in brief, which will help the researcher to easily choose an appropriate analytical technique for quantification of their drugs of interest.

2 Modern Analytical Techniques for Bioanalysis

Chromatography is one of the basic analytical techniques used for the separation of a pure component from a mixture. But now many changes and developments have taken place and it is widely used in pharmaceutical industries around the globe. It works on the principle of adsorption or partitioning between two phases. Chromatography can be of different types, such as gas chromatography, liquid chromatography, supercritical fluid chromatography, capillary liquid chromatography with UV detector, thin layer chromatography, etc. Each has separate applications along with advantages and disadvantages. Among majorly used chromatographic techniques, HPLC/ultra high-performance liquid chromatography (UHPLC) is highly reliable, easy to maintain and has good performance. It can be useful in high throughput screening in drug discovery and in a pharmacokinetic study of the drug. Preparative HPLC is used for isolation of components from a mixture for their identification and structure elucidation. HPLC is also used for metabolomics studies of various agents as well.

Nowadays, MS is a widely used technique for bioanalysis in which sample molecules get ionized and are separated based on their mass to charge ratio. Different ionization techniques employed in MS include electrospray ionization (ESI), chemical ionization, atmospheric pressure photoionization, atmospheric pressure chemical ionization (APCI), and matrix-assisted laser desorption ionization (MALDI). Various analyzers used in MS include quadrupole, time of flight (TOF), and ion trap. The detector used is either an electron multiplier or Faraday cup detector. Because of their higher sensitivity and speed of scanning, high resolution mass spectrometers are mostly applied in analyzing peptides in endogenous biomarker detection and bioanalysis of complicated molecules. A sequential arrangement of both LC and MS gives the advantage of using both simultaneously as a more influential and resourceful instrument. This combination turns out to be a great analytical tool and is known as “hyphenated” techniques. Not only LC-MS but also other advanced hyphenated techniques, like liquid chromatography–nuclear magnetic resonance (LC-NMR), GC-MS, and LC-MS-NMR, are used for qualitative or quantitative bioanalysis in drug discovery and drug development. The hyphenated techniques provide for special application in bioanalysis and identification of in vitro and in vivo metabolites. NMR can be used for checking the purity of the compounds with the advantage of easy sample preparation and short analysis time.

3 Sample Treatment

Sample treatment improves the sensitivity of a method by concentrating the analyte in the sample. The method of treatment is selected based on the physicochemical properties of the drug, type of matrices and detection method. Different methods of sample preparation are used for different matrices, such as blood, peripheral blood mononuclear cells (PBMC), etc.
cells, and tissues. A scientist must remove matrix and other interferences from a sample. Most widely used sample preparation techniques are protein precipitation (PPT), liquid–liquid extraction (LLE), and solid phase extraction (SPE). Recovery is different in all three techniques. The difference between the areas of extracted samples and unextracted standard gives the recovery of analyte in the matrix. SPE is better than the other two techniques in terms of recovery but it is costlier compared to the other techniques. LLE is suitable for extracting non-polar drugs from the matrix as it can extract the drug using organic immiscible solvents, like ethyl acetate, diethyl ether, tertiary butyl methyl ether, and hexane. PPT is mostly used when the analyte of interest is present in urine. The sample preparation techniques are selected based on simplicity, cost, and robustness. When a trace amount of a sample has to be detected, clean-up is a critical step to ensure higher recovery and sensitivity. Effects of interferences and matrices are also influenced by clean-up effectiveness. Figure 1 schematically shows the major steps to be followed for extraction of analytes from biological matrices by LLE, PPT and SPE techniques.

3·1 Liquid-liquid extraction
LLE is a conventional technique used for extraction of an analyte from biological samples. It works on the principle of partitioning between the matrix, which contains a mixture of polar and non-polar solvent. In LLE, two immiscible phases are used. After mixing of the phases, they are separated by using centrifugation, freezing or a semi-permeable membrane. The non-polar phase is selected based on the solubility and polarity of the sample. Extraction capability of the solvent can be altered by adding another solvent having a different lipophilicity. The major disadvantage of LLE includes lack of automation. However, nowadays, a semi-automated technique with automation in liquid handlers is used for the separation of the two phases. It is trustworthy and also gives a clean sample. Generally, with the choice of an effective extracting solvent, LLE results in more than 80% recovery.

3·2 Protein precipitation
PPT is the method in which a reaction between a protein entity and solvent has taken place. By providing acidic or alkaline conditions, the ionize state of a protein can be shifted. Concentrated salts also can change the hydration state of a protein, which results in a decrease in their solubility. In addition to the non-polar solvents and salts, some detergents also decrease the hydration state of proteins. For analysis of drugs in biological matrices, PPT is performed to remove larger protein groups. One of the ways to prevent loss of analyte using the PPT method is to use water-miscible organic solvent. The amount of protein being eliminated is based on the solvent and its quantity used for extraction. Sometimes, refrigeration also increases the formation of protein precipitates.

3·3 Solid phase extraction
SPE is a relatively advanced technique compared to LLE and PPT. In an earlier time, small sized columns prepared in the
laboratory itself were used but the method was not well known. This technique became better known after the accessibility of packed cartridges. In these cartridges, silica is packed with stationary phase as in chromatographic columns. The principle is similar to that of chromatography, i.e. affinity of an analyte with stationary phase. There are many advantages of choosing SPE over other extraction techniques. The major ones include the possibility of automation, better extraction efficiency, use of smaller amounts of solvents, availability of wide varieties of stationary phase for different types of analytes and simultaneous clean-up. The proper SPE device is selected based on the parameters, such as sample quantity, contaminants present, and a complication of matrix and quantity of analyte.24

3-4 Advanced sample extraction techniques

Nowadays, other methods of extraction have also been introduced, such as solid phase microextraction (SPME), dried blood spotting (DBS), supercritical fluid extraction (SCFE), matrix solid-phase dispersion (MSPD) and hybrid technique. SPME is a simple technique that completely avoids the use of solvents. It works on the simple principle of partitioning between the matrix and stationary phase. The advantage of SPME over SPE includes its higher detection level, whereas disadvantages include a problem in the analysis of polar and non-volatile compounds.25

Another advancement in sample preparation techniques is DBS, under which a small amount of blood is collected on a piece of paper and dried. For analysis, a small part of the paper is taken, and blood is separated from it using phosphate buffer solution, from which further dilutions are made for analysis. The advantages over other techniques include the requirement for a smaller volume of blood (around 100 µL) for analysis, less invasive, and easy to store and transport.26

SCFE involves extraction of a component from the matrix with the help of SCF as a solvent for extraction. The fluid used for extraction is carbon dioxide, modified methanol, ethanol, etc. They are used above critical temperature and pressure for proper extraction. SCFE ensures complete extraction and further, it can be separated easily by reducing temperature or pressure. Also, it is a faster process and the fluids used are less costly.27

MSPD is an extraction technique that can be used for the extraction of solid, semi-solid or viscous materials. MSPD involves admixing of the sample matrix with the solid support, like silica with organic groups attached to its surface. MSPD silica works as a blender to divide solid particles into small parts. The sample particles get distributed in the organic phase. Distribution takes place based on their polarities. The advantage of this technique includes its capability in the quantification of an analyte from a very small amount of sample.28

4 Analysis of Anticancer Drugs Using Different Biomatrices

4-1 Plasma

Plasma is the most important and widely used biological matrix for bioanalysis. The advantage of plasma over other biological matrices is that it is easily available, cheap and gives a proper indication of drug amount in blood. If the concentration of drug is low in blood, then plasma will be a better choice as there is a higher recovery rate in plasma than any other matrix. If the drug has higher protein binding, then plasma is one of the matrices of choice. In the case of animal studies, measurement of plasma drug concentration is preferred because of small sample size. The advantage of using plasma over serum is that there is less chance of losing a sample in plasma. Plasma is collected after the centrifugation of the blood containing anticoagulant and collection of the resulting supernatant. Plasma is produced when whole blood is collected in tubes that are treated with an anticoagulant. For quantification of acalabrutinib in rat plasma, a method was developed by Surendran et al. where extraction was performed using the SPE method and an Eclipse Plus C8 column was used. The mobile phase was a mixture of 10 mM ammonium formate and acetoniitrile in gradient run. The mass spectrometer was equipped with an ESI source with triple quadrupole mass analyzer in which detection was done with positive ion in multiple reaction monitoring (MRM) mode.29

Sparidans et al. developed a method for quantification of crizotinib in mouse plasma using the PPT method of extraction. Separation was achieved by a BEH C18 column, which was maintained at 40°C. The mobile phase consisted of a mixture of 0.1% ammonium hydroxide and methanol. The mass analysis was performed using ESI in positive ionization mode with triple quadrupole in single reaction monitoring (SRM) mode. Total analysis time was 2.5 min.30 Another method for crizotinib quantification was developed by Qiu et al. in rat plasma using PPT as a method of extraction using a Zorbax XDB C18 column for separation. A premix mobile phase of water and methanol with 0.1% formic acid was used. For mass analysis, ionization was carried out with ESI in positive mode and analysis was conducted with triple quadrupole in MRM mode.31

Zheng et al. developed a method for erlotinib and vemurafenib quantification in plasma of cancer patients. LLE was used to extract the analyte from the plasma samples. Chromatographic separation was achieved using a C8 Xterra MS column maintained at 50°C. The mobile phase was a mixture of glycine buffer and acetonitrile in a ratio of 45:55 (%, v/v). Detection was performed using a dual wavelength UV detector at 331 nm for erlotinib and 249 nm for vemurafenib, and the total run time was 12 min.32

A method for ibrutinib quantification in human and mouse plasma was developed by Rood et al. where pretreatment was carried out using the PPT method. Separation was achieved using an XBridge BEH300 column set at 40°C. The mobile phase comprised of 0.1% formic acid in water and methanol. The mass analysis was performed using the ESI interface in positive ionization mode with quadrupole mass analyzer in SRM mode. Total run time was 3 min.33

Spatari et al. developed a method for quantification of Lorlatinib in mouse plasma using PPT as a method of extraction and separation was achieved using a Varian Polaris C18-A column maintained at 40°C. The mobile phase had a gradient of 0.1% formic acid in water and methanol. For mass analysis, ionization was carried out with ESI in positive ion mode and analysis was conducted with triple quadrupole in SRM mode.34

A method for midostaurin quantification in the plasma of patients having advanced systemic mastocytosis was developed by Bourget et al. in which PPT was used as the extraction technique. A Sunfire C18 silica column maintained at 30°C was used with a premixed mobile phase consisting of 10 mM ammonium formate in water and acetonitrile with 0.1% formic acid. The mass analysis was performed using ESI interface in positive ionization mode with quadrupole mass analyzer in SRM mode.35

Wani et al. developed a method for neratinib quantification in human plasma in which the method of extraction was PPT and separation was achieved using a UPLC BEH C18 column. The mobile phase consisted of a mixture of methanol, water and
formic acid in 70:30:0.1 (%, v/v) ratio. For mass analysis, ionization was done with ESI in positive mode and analysis was conducted with triple quadrupole in MRM mode. A method for rucaparib quantification in human and mouse plasma was developed by Sparidans et al., where the sample was pre-treated with acetonitrile for precipitating proteins and further separation was achieved using a Polaris 3 C18 A column maintained at 40°C. A mixture of 0.02% formic acid and methanol was used as the mobile phase. The mass analysis was performed using ESI interface in positive ion mode with quadrupole mass analyzer in SRM mode. Total analysis time was 3 min.37

Hidau et al. developed a method for talazoparib quantification in rat plasma using PPT as a method of extraction and a Luna C18 column for separation. The mobile phase had isocratic elution of methanol:water in 60:40 ratio and acetonitrile:water in 65:35 ratio. The detector used was UV set at 227 nm. Total chromatographic runtime was 10 min.38 Details of different bioanalytical method parameters reported for the analysis of anticancer drugs using plasma as a biological matrix have been summarized in Table 1.

### 4.2 Serum

Serum is considered to be one of the important biological matrices for bioanalysis. The composition of serum is the same as plasma except for the absence of the fibrinogen and other clotting factors in the serum. Serum is collected after the clotting of the blood, removal of the clot by centrifugation and collection of the resulting supernatant. Serum drug concentration is generally measured in the case of drugs requiring therapeutic drug monitoring, which is either anticancer or antibiotics. It is also important as it gives the indication of the toxic effect of a particular level of drug in the body. Sometimes, measurement of serum drug concentration becomes of utmost importance; particularly when the patient shows great variability in drug pharmacokinetics, if it is a prophylactic drug or when a drug is found to develop resistance. The advantage of serum is that it is free from platelets and other cellular elements, which have the potential to alter bioanalytical results, and it is more stable in comparison to plasma during storage.39 Shu et al. developed an LC-MS/MS method for the simultaneous quantification of thalidomide, lenalidomide, cyclophosphamide, bortezomib, dexamethasone and Adriamycin in serum of multiple myeloma patients (MM). The extraction process was SPE and the column used was a BEH C18 kept at 30°C. The mobile phase was composed of a mixture of aqueous solution of 0.1% formic acid and acetonitrile. In the mass spectrometric analysis, ionization was performed using ESI in positive mode and detection was achieved in MRM.40

Nielka et al. developed a method for quantification of imatinib, sunitinib, nilotinib, dasatinib, pazopanib, and regorafenib in human serum. The extraction technique used was PPT and separation was achieved by a BEH C18 column (50°C). Elution was achieved with a premixed mobile phase containing water and methanol with 0.1% formic acid and 2 mM ammonium acetate. Total analysis time was 7 min. For mass analysis, the ionization source was ESI in positive mode and detection was performed using a triple quadrupole detector in MRM mode.41

In the year 2017, Rodriguez et al. developed a method for the separation and quantification of erlotinib in the human serum of healthy volunteers and patients receiving erlotinib treatment. The drug and its metabolites were separated using methanol and phosphate buffer (60:40) as mobile phase with a C-18 column at 248 nm in HPLC, using diode array detector (DAD).42 In the same year, Suga et al. developed a method for erlotinib quantification in human serum where the drug was extracted using LLE in t-butyl methyl ether. Separation was achieved using an Inertsil ODS-3 analytical column and the ratio of mobile phase used was 74:26 potassium phosphate buffer and acetonitrile. The column temperature was 60°C and the analyte was detected at the UV wavelength of 345 nm in UHPLC. The developed method was employed for the determination of erlotinib in serum of a non-small cell lung cancer patients.43 For imatinib quantification in human serum, Marek et al. developed an HPLC method in which extraction from serum was performed using an Extrelut NT 3 extraction tube. The column used was a Lichrospher 100-5 RP8 and detection was achieved using DAD at 265 nm. A mixture of potassium dihydrogen phosphate and acetonitrile (7:3) was used as the mobile phase in isocratic mode. The total run time for analysis was 15 min.44 Later, one more method was developed by Marcondes et al. for the quantification of imatinib in human serum employing PPT as the sample extraction technique. The column used was a Luna C-18 column kept at 40°C.

| Compound       | Analytical techniques | Extraction technique | Column, mobile phase, elution mode | Detection technique | Sample matrix | Quantification range | Reference      |
|----------------|-----------------------|----------------------|-----------------------------------|---------------------|---------------|---------------------|-------------------|
| Acalabrutinib  | LC-MS/MS              | SPE                  | Eclipse plus C8, HCOOHBHs and ACN, gradient | Triple quadrupole in MRM mode | Rat plasma | 0.20 - 149.35 ng/mL | 29               |
| Crizotinib     | LC-MS/MS              | PPT                  | BEH C18 column, NLOH and CH.OH, gradient | Triple quadrupole in SRM mode | Mouse plasma | 10 - 8000 ng/mL    | 30               |
| Lorlatinib     | LC-MS/MS              | PPT                  | XDB C18 column, water and CH.OH with HCOOH gradient | Triple quadrupole in SRM mode | Rat plasma | 1 - 2000 ng/mL     | 31               |
| Erlotinib      | HPLC-UV               | LLE                  | CX Xterra MS column, glycine buffer and ACN, isocratic | UV at 331nm | Cancer patient’s plasma | Human and mouse plasma | 50 - 4000 ng/mL | 32               |
| Brevunib       | LC-MS/MS              | PPT                  | XBridge BEH300 column, HCOOH in water and CH.OH, gradient | Quadrupole in SRM mode | Cancer patient’s plasma | Human plasma | 5 - 4000 ng/mL | 33               |
| Lorlatinib     | LC-MS/MS              | PPT                  | Varian Polaris C18-A column, HCOOH in water and CH.OH, gradient | Triple quadrupole in SRM mode | Mouse plasma | 2 - 2000 ng/mL     | 34               |
| Midostaurin     | LC-MS/MS              | PPT                  | SunFire C18 silica column, HCOONH, in water and HCOOH in ACN, gradient | Triple quadrupole in SRM mode | Human plasma | 75 - 2500 ng/mL    | 35               |
| Neratinib      | UPLC-MS/MS            | PPT                  | UPLC BEH C18 column, CH.OH, water and HCOOH, isocratic | Triple quadrupole in SRM mode | Human and mouse plasma | Human plasma | 4 - 500 ng/mL | 36               |
| Rucaparib      | LC-MS/MS              | PPT                  | Polaris 3 C18-A column, HCOOH and CH.OH, gradient | Triple quadrupole in SRM mode | Human and mouse plasma | Human plasma | 1.25 - 2000 ng/mL | 37               |
| Talazoparib    | HPLC-UV               | PPT                  | Luna C-18 column, CH.OH/ACN and deionized water, isocratic | UV at 227nm | Rat plasma | 100 - 2000 ng/mL | 38               |
| Vemurafenib    | HPLC-UV               | LLE                  | CX Xterra MS column, glycine buffer and ACN, isocratic | UV at 249nm | Cancer patient’s plasma | Human plasma | 1.25 - 100 μg/mL | 32               |
Table 2  Bioanalytical methods for the analysis of anticancer drugs in serum

| Compound               | Analytical techniques | Extraction technique | Column, mobile phase, elution mode | Detection technique | Sample matrix | Quantification range | Reference |
|------------------------|-----------------------|----------------------|-----------------------------------|--------------------|---------------|----------------------|-----------|
| Bortezomib             | LC-MS/MS              | SPE                  | BEH C18 column, HCOOH and ACN, gradient | Triple quadrupole in MRM mode | Serum of MM patients | 2 - 1000 ng/mL       | 40        |
| Cyclophosphamide       | LC-MS/MS              | SPE                  | BEH C18 column, HCOOH and ACN, gradient | Triple quadrupole in MRM mode | Serum of MM patients | 2 - 2000 ng/mL       | 40        |
| Dasatinib              | LC-MS/MS              | SPE                  | BEH C18 column, HCOOH and ACN, gradient | Triple quadrupole in MRM mode | Human serum    | 5 - 400 ng/mL         | 41        |
| Dexamethasone          | LC-MS/MS              | SPE                  | BEH C18 column, HCOOH and ACN, gradient | Triple quadrupole in MRM mode | Serum of MM patients | 2 - 500 ng/mL        | 40        |
| Doxorubicin            | LC-MS/MS              | SPE                  | BEH C18 column, HCOOH and ACN, gradient | Triple quadrupole in MRM mode | Serum of MM patients | 2 - 2000 ng/mL       | 40        |
| Erlotinib              | HPLC                  | NR                   | C18 column, CH₃OH and PO₄³⁻ buffer, isoctratic | DAD at 248 nm | Human serum | 16.80 - 10¹⁰ ng/mL   | 42        |
| Imatinib               | UHPLC-UV              | LLE                  | Inertial OD-3 column, KH₂PO₄ and ACN | UV at 345 nm | Human serum | 6 - 6000 ng/mL        | 43        |
|                        | HPLC                  | NR                   | Lichrospher 100-5 RP8 column, KH₂PO₄ and ACN | DAD at 265 nm | Human serum | 0.03 - 10 µg/mL      | 44        |
|                        | LC-MS/MS              | SPE                  | BEH C18 column, HCOOH and ACN, gradient | Triple quadrupole in MRM mode | Human serum | 0.1 - 4 µg/mL         | 41        |
|                        | LC-MS/MS              | PPT                  | Luna 100 Å C-18 column, CH₃OH and HCOOH gradient | Single quadrupole analyzer | Human serum | 0.50 - 10 µg/mL       | 45        |
| Lenalidomide           | LC-MS/MS              | SPE                  | BEH C18 column, HCOOH and ACN, gradient | Triple quadrupole in MRM mode | Human serum | 2 - 1000 ng/mL        | 40        |
| Lenvatinib             | LC-MS/MS              | LLE                  | Symmetry shield RP8 column, CH₃COONH₄ and ACN, isoctratic | Triple quadrupole in MRM mode | Human serum | 0.08 - 400 ng/mL      | 46        |
| Nilotinib              | LC-MS/MS              | PPT                  | BEH C18 column, HCOOH and CH₃COONH₄ gradient | Triple quadrupole in MRM mode | Human serum | 0.1 - 4 µg/mL         | 41        |
| Pazopanib              | LC-MS/MS              | PPT                  | BEH C18 column, HCOOH and CH₃COONH₄ gradient | Triple quadrupole in MRM mode | Human serum | 1 - 40 µg/mL          | 41        |
| Regorafenib            | LC-MS/MS              | PPT                  | BEH C18 column, HCOOH and CH₃COONH₄ gradient | Triple quadrupole in MRM mode | Human serum | 0.1 - 4 µg/mL         | 41        |
| Sorafenib              | HPLC                  | LLE                  | Pinnacle DB C18 Column, KH₂PO₄ and ACN, isoctratic | UV at 260 nm | Human serum | 50 - 5000 ng/mL       | 47        |
| Sunitinib              | LC-MS/MS              | PPT                  | BEH C18 column, HCOOH and CH₃COONH₄ gradient | Triple quadrupole in MRM mode | Human serum | 2 - 180 ng/mL         | 41        |
| Thalidomide            | LC-MS/MS              | SPE                  | BEH C18 column, HCOOH and ACN, gradient | Triple quadrupole in MRM mode | Serum of MM patients | 4 - 1000 ng/mL       | 40        |
|                        | LC-MS/MS              | LLE                  | Prodigy C18 column, ACN and water, isoctratic | Triple quadrupole in MRM mode | Human serum | 0.05 - 20 µg/mL       | 48        |

a. Not reported.

The analyte was analyzed using a mobile phase consisting of methanol and water having 10 mM ammonium acetate and 0.1% formic acid. The mass analysis was performed in positive ionization mode with ESI interface.45

For lenvatinib, a method was developed in human serum by Mano et al. in which the extraction technique was LLE and the column used for separation was a Symmetry Shield RP8 maintained at 40°C. An isotropic mobile phase containing a mixture of ammonium acetate and acetonitrile in 60:40 ratio was used. For mass spectrometry, ionization was performed using ESI and detection was carried out using triple quadrupole mass analyzer in MRM mode.46

For sorafenib quantification, Heinz et al. developed an HPLC method in human serum in which the extraction was performed using the LLE technique and separation was achieved using a Pinnacle DB C18 column. The mobile phase used was potassium dihydrogen phosphate and acetonitrile (35:65) in isoctratic elution mode. The detection was performed at 260 nm using a UV detector and the total analysis time was 10 min.47

Sandra et al. developed a method for thalidomide quantification in human serum. The extraction was carried out using the LLE technique and the separation was performed with a Prodigy C18 column maintained at 20°C. The premixed mobile phase of acetonitrile and water (3:7) containing 0.1% formic acid was used in isoctratic mode. The total analysis time was 13 min. In mass spectrometry, ESI was used for ionization with triple quadrupole mass analyzer in MRM mode.48 Details of different bioanalytical method parameters reported for the analysis of anticancer drugs using serum as a biological matrix have been summarized in Table 2.

4.3 PBMC
PBMCs are a group of immune cells having round nuclei, lymphocytes, monocytes and dendritic cells. They can easily be isolated from whole blood using different methods. PBMC is used as a matrix for the analysis of drugs which are activated inside the cells by a specific enzyme or for drugs having a site of action inside the cells. For example, the target of action of all anti-human immuno deficiency virus (anti-HIV) drugs is T-lymphocyte.49 Therefore, it is necessary to analyze anti-HIV drugs in T-lymphocyte, which is a part of PBMCs. Some anticancer drugs, like gemcitabine, phosphorylate into its active form inside the cell.50

For dasatinib a quantification method in PBMC was developed by D’Avolio et al. in which PBMCs were isolated using density gradient centrifugation. Separation was achieved using an Atlantis T3 C18 column maintained at 35°C and the mobile phase in gradient elution consisted of acetonitrile and water, both containing 0.05% formic acid. The mass detector used was a Micromass ZQ mass detector operated in ESI positive ionization with single ion recording (SIR) mode.51 Renouleau et al. developed a method for everolimus quantification in human PBMC. Extraction was performed using the PPT technique. Chromatographic separation was achieved using a MassTrak TDM C18 column maintained at 55°C with water and methanol in gradient as a mobile phase where both had 0.1% formic acid and 2 mM ammonium acetate. The mass analysis was performed using an ESI source in positive mode and it was monitored in MRM mode.52

Huang et al. developed a method for fludarabine quantification in human PBMCs using a Hypercarb column for separation in
gradient elution using a mobile phase of 100 mM ammonium acetate and acetonitrile. Mass analysis was carried out using ESI in negative mode and monitored in MRM mode.53

Gemcitabine which is another anti-metabolite is used against various solid tumors. For gemcitabine, Veltkamp et al. developed a method for quantification in human PBMCs using a BioBasic AX ion exchange column. The mobile phase step gradient of phase-A having 10 mM ammonium acetate in acetonitrile and water of pH 6 and phase-B having 1 mM ammonium acetate in acetonitrile and water pH 10.5 was used. The total chromatographic run was 10 min. For mass analysis, turbo ion spray was used in negative ion mode using a triple quadrupole mass analyzer.50

A quantification method for imatinib in PBMCs was developed by D’Avolio et al. The extraction was done by LLE method and separation was achieved using a Luna C18 column maintained at 65°C. A mixture of methanol and 5 mM ammonium acetate having 0.01% of TFA at pH 3.2 was used as the mobile phase and the total chromatographic run time was 10.5 min. In the mass analysis, turbo electrospray was used as an ion source in positive ion mode. The analysis was carried out using a triple quadrupole mass analyzer in MRM mode.55

For lenvatinib quantification in whole blood, Dubbelman et al. developed a method using LLE and separation with a Symmetry Shield RP8 column maintained at 40°C with mobile phase having 2 mM ammonium acetate and acetonitrile (60:40). The total run time was 12 min. The mass spectrometer used ESI in positive ion mode. The analyzer was triple quadrupole operated in MRM mode.56 Details of different bioanalytical method parameters reported for the analysis of anticancer drugs in whole human blood have been summarized in Table 3.

### 4-5 Urine

One of the reasons to choose urine as a biological sample is the presence of a high concentration of the analyte and metabolites for the majority of drugs contained. Urine is a major route of excretion for all drugs. Commonly, a urine sample is checked for identifying the metabolites of any drug. Where urinary excretion is the primary route of drug elimination, there must be a validated method for bioanalysis of drugs and its metabolites in urine.57 Each person has a different pharmacokinetic rate and to understand the adsorption, distribution, metabolism, excretion and toxicity of any drug, its determination in urine along with plasma is required.58 Advantages of urine as a matrix are the requirement of easy collection and sample preparation procedure, and less interference from matrix components leading to less complicated analysis.

A method for quantification of afatinib, tofacitinib and cabozantinib in human urine was developed by Adnan et al. where extraction was done using the LLE method and separation was achieved in a Luna C18 column. An isocratic mobile phase mixture containing acetonitrile and 0.01 M ammonium formate buffer (5:5) was used. Mass spectrometric analysis using an ESI ion source in positive mode with a triple quadrupole mass analyzer was carried out.59

### Table 3 Bioanalytical methods for the analysis of anticancer drugs in human PBMC and whole human blood

| Compound       | Analytical technique | Extraction technique | Column, mobile phase, elution mode | Detection technique | Sample matrix | Quantiﬁcation range | Reference |
|----------------|----------------------|----------------------|------------------------------------|---------------------|---------------|----------------------|-----------|
| Dasatinib      | HPLC-MS              | LLE                  | Atlantis T3 C18 column, ACN and with HCOOH, gradient | Micromass ZQ mass detector | Human PBMC    | 0.25 – 8 ng          | 51        |
| Everolimus     | LC/MS/MS             | PPT                  | MassTrak TDM C18 column, water and CH-OH, gradient | Triple quadrupole in MRM mode | Human PBMC    | 1.25 – 10 ng/mL      | 52        |
| Fludarabine    | LC/MS/MS             | NR*                  | Hypercarb Column, ammonium acetate and ACN gradient | Triple quadrupole in MRM mode | Human PBMC    | 1 – 40 ng/mL         | 53        |
| Gemcitabine    | LC/MS/MS             | NR*                  | BioBasic AX ion exchange column, ammonium acetate in ACN and water, gradient | Triple quadrupole mass analyzer | Human PBMC    | 1 – 25 ng/mL         | 50        |
| Imatinib       | HPLC-MS              | LLE                  | Atlantis T3 C18 column, ACN and water with HCOOH, gradient | Micromass ZQ mass detector | Human PBMC    | 0.25 – 8 ng          | 51        |
| Nilotinib      | HPLC-MS              | LLE                  | Atlantis T3 C18 column, ACN and water with HCOOH, gradient | Micromass ZQ mass detector | Human PBMC    | 0.25 – 8 ng          | 51        |
| Everolimus     | LC/MS/MS             | LLE                  | Nucleosil C18 column, ACN and CH3COONH4, isocratic | Triple quadrupole in MRM mode | Whole human blood | 0.368 - 205 ng/mL    | 54        |
| Imatinib       | LC/MS/MS             | PPT                  | Luna C18 column, CH3OH and CH3COONH4 gradient | Triple quadrupole in MRM mode | Whole human blood | 0.03 – 75 ng/mL      | 55        |
| Lenvatinib     | LC/MS/MS             | LLE                  | Symmetry shield RP8 column, ACN and CH3COONH4, gradient | Triple quadrupole in MRM mode | Whole human blood | 0.25 - 500 ng/mL     | 56        |
| a. Not reported. |                      |                      |                                    |                     |               |                     |           |
Dubbelman et al. developed a method for the analysis of bendamustine and its metabolite. The SPE technique and a Synergi column along with mobile phase containing 5 mM ammonium formate having 0.1% formic acid and methanol were used. The mass spectrometer was equipped with ESI interface in positive mode and the analysis was carried out using the MRM technique with triple quadrupole mass analyzer. Another method for bendamustine quantification in human urine was developed by Plenis et al. using LLE as the sample extraction method and a Synergi Max RP column for separation.

Caboantinib was developed by Marahatta et al. for bendamustine quantification in human urine using the SPE extraction method and a Synergi column along with mobile phase containing 5 mM ammonium formate having 0.1% formic acid and methanol, gradient.

Another method for bendamustine quantification in human urine was developed by Plenis et al. using LLE as the sample extraction method and a Synergi column for separation.

Another method for bendamustine quantification in human urine was developed by Marahatta et al. using the SPE extraction method and separation in an Agilent TC-C18 column at 30°C. The mobile phase consisted of a mixture of acetonitrile and potassium dihydrogen phosphate (32:68). The HPLC analysis used fluorescence detector. Marahatta et al. developed another method for bendamustine quantification in human urine by using a fused silica capillary maintained at 25°C. The optimized mobile phase was a mixture of acetonitrile and water (25:75).

Another method for quantification of cyclophosphamide along with ifosfamide, doxorubicin, epirubicin and daunorubicin in human urine was developed by Sottani et al. Extraction technique used was the SPE extraction method and separation was achieved using a BDS Hypersil C8 column. The mobile phase had 0.1% formic acid and acetonitrile. Total analytical run time was 15 min. The mass spectrometer was equipped with ESI in positive mode. It was monitored in MRM mode with a triple quadrupole mass analyzer.

Another method for quantification of cyclophosphamide along with ifosfamide, doxorubicin, epirubicin and daunorubicin in human urine was developed by Sottani et al. Extraction technique used was the SPE extraction method and separation was achieved using a BDS Hypersil C8 column. The mobile phase was a mixture of acetonitrile and water (25:75). Total analytical run time was 15 min. The mass spectrometer was equipped with ESI in positive mode. It was monitored in MRM mode with a triple quadrupole mass analyzer.

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Another method for quantification of cyclophosphamide along with ifosfamide, doxorubicin, epirubicin and daunorubicin in human urine was developed by Sottani et al. Extraction technique used was the SPE extraction method and separation was achieved using a BDS Hypersil C8 column. The mobile phase was a mixture of acetonitrile and water (25:75).

Another method for quantification of cyclophosphamide along with ifosfamide, doxorubicin, epirubicin and daunorubicin in human urine was developed by Sottani et al. Extraction technique used was the SPE extraction method and separation was achieved using a BDS Hypersil C8 column. The mobile phase was a mixture of acetonitrile and water (25:75). Total analytical run time was 15 min. The mass spectrometer was equipped with ESI in positive mode. It was monitored in MRM mode with a triple quadrupole mass analyzer.
The mobile phase consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. Total chromatographic run time was 21 min. For mass analysis, detection was done in MRM with a triple quadrupole mass analyzer operated in ESI positive mode. Anetal et al. developed a method for niraparib quantification in human urine. Urine was diluted with acetonitrile and methanol. A SunFire C18 column with column maintained at 40°C and mobile phase having 20 mM ammonium acetate and formic acid, acetonitrile and methanol (0.1:50:50) in gradient mode was employed. The mass spectrometer used ESI in positive mode and triple quadrupole mass analyzer in MRM mode for detection. Wang et al. developed a method for ponatinib quantification in the urine samples of Sprague-Dawley rats where extraction was performed using LLE and separation was done using a Cortecs column kept at 40°C. The mobile phase was comprised of water with 0.1% formic acid and 2 mM ammonium formate and methanol. The mass analysis was performed using ESI positive mode ionization and detected in MRM mode.

For sorafenib quantification in human urine, Sreedhar et al. developed a method using DPV at dropping mercury electrode. Table 4 summarizes the details of different bioanalytical method parameters reported for the analysis of anticancer drugs in urine.

4-6 Tissue
A tissue distribution study is one of the essential elements of a pharmacokinetic study in the drug development stage. Sometimes, pharmacokinetic parameters determined using plasma samples may not clearly indicate the action and efficacy of the drug on targeted tissue. The amount of drug entering the tissues helps in finding the kinetically active drug and fixing the dose of a drug. Again, plasma drug concentration cannot resolve the kinetics of drugs targeting the brain. A tissue distribution study also helps in finding drug toxicity at particular tissues. Different tissue lysis techniques are used for sample preparation in tissue studies like grinding, sonication, and pulverizing, and the sample is extracted using sample extraction techniques such as PPT, LLE or SPE. The sample is then analyzed by an instrument, like LC-MS/MS, GC-MS, or LC-UV.

Sparidans et al. developed an LC-MS/MS method for quantification of alectinib, brigatinib and lorlatinib in mouse liver, kidney, spleen and brain tissue homogenates. The method used for extraction was PPT and the column used for separation was a BEH C18 column maintained at 40°C. The mobile phase used was 1% formic acid in water and acetonitrile in gradient elution mode. For mass analysis, the ion source used was turbo electrospays in positive ion mode and it was monitored in SRM mode using a triple quadrupole mass analyzer. Feng et al. developed a method for quantification of apatinib in various tissues of mice. The method used for extraction was PPT and the separation was achieved using a Zorbax SB-C18 column. The mobile phase had 0.1% formic acid in water as an aqueous phase and methanol as an organic phase in gradient run for 7.5 min. A triple quadrupole mass analyzer with ESI ionizer in positive mode was used for mass detection. The drug was found to be majorly distributed in the intestine, liver, kidney, heart, lung and spleen.

For AZD3759, Xiong et al. developed a method for quantification in brain tissue. The extraction was done using the PPT method. The separation was performed using Hypersil a GOLD C18 column kept at 25°C with acetonitrile and 0.1% formic acid in water as the mobile phase in linear gradient elution mode. The total analysis time was 3.5 min. The mass analysis was done in single reaction monitoring where the ionization source was ESI in positive ion mode.

For bendamustine, He et al. developed a method for quantification in mouse brain tissue. The extraction procedure was PPT using methanol. The column used for separation was a Zorbax C18 and water and methanol containing 0.1% formic acid was used as the mobile phase. The total run time was 8.2 min. The mass ionization was done using ESI in positive mode and it was monitored in SRM mode. A method for caboctinib was developed by Wang et al. in which separation was achieved using an UPLC BEH C18 column maintained at 40°C and mobile phase consisting of a mixture of acetonitrile and water with 0.1% formic acid. The total chromatographic run was of 3 min. The mass analysis was carried out using a triple quadrupole mass analyzer in MRM mode using ESI ionization in positive mode. The tissue distribution was found to be maximum in the liver and minimum in the heart.

Teoh et al. developed a method for quantification of imatinib in various tissues of mouse where extraction was performed using the PPT method. Separation was achieved using an Inertsil CN-3 column and a mobile phase ratio of water(64): methanol(35):triethylamine(1) in isocratic elution. Later, another method for quantification of imatinib in heart and liver tissue of rats was developed by Bianchi et al. Extraction was carried out using the solvent extraction method and separation was achieved using a C18 Waters column maintained at 30°C. The mobile phase consisted of a mixture of 0.2% formic acid in water and 0.2% formic acid in methanol. For mass analysis, an ion trap analyzer with ESI mode was used. It was monitored in SRM mode.

For LBH589 quantification in mouse tissues, a UPLC-MS/MS method was developed by Mendoza et al. The extraction was done using LLE and separation was achieved using a UPLC BEH column set at 20°C. The mobile phase consisted of a mixture of water and methanol (10:90) in isocratic elution where 0.1% TFA was added in both phases. For MS detection, ESI was used as ionization source and operated in positive mode, and the analyzer used was triple quadrupole in MRM mode.

A method for determination of pazopanib in brain tissue of mouse was developed by Minocha et al. using the LLE technique and an X Terra C18 column for separation. Acetonitrile and water with 0.1% formic acid in a ratio of 7:3 were used as mobile phase and total run time was 3.5 min. The mass spectrometer was operated with ion spray source in positive ion mode. The analyte was monitored in MRM mode.

For quantification of pomalidomide in mouse brain tissue, Jiang et al. developed a method in which extraction was achieved using the PPT method with acetonitrile. Separation was done with a Zorbax C18 column and the mobile phase of water (having 0.1% formic acid) and acetonitrile in a linear gradient in a total run time of 10 min. APCI source with triple quadrupole mass analyzer in SRM mode was used for mass detection.

Sparidans et al. developed a method for the quantification of sorafenib in liver homogenate. The extraction was performed using PPT and the separation was achieved using a Polaris-3 C18 column kept at 40°C. The mobile phase used was 0.1% formic acid in water and acetonitrile in gradient elution mode. Mass analysis was performed using ESI in positive ionization mode detected by a triple quadrupole mass analyzer in MRM mode.

Zhou et al. developed a method for sunitinib using the LLE technique for extracting from brain tumor tissue and SPE for extracting from normal brain tissue. Separation was achieved using a Luna C8 column maintained at 30°C. The isocratic mobile phase was composed of acetonitrile and 1 mM
ammonium acetate with 0.1% acetic acid (28:72). Total chromatographic run time was 3.2 min. The mass detection was performed with ESI ion source in positive ion mode, which was monitored in MRM mode using a triple quadrupole mass analyzer.\^\textsuperscript{83} Later in the year 2013, another method for quantification of sunitinib in brain tissue homogenate of mouse ICR mice tissue was developed by Ma et al. \textsuperscript{84} where the LLE technique is used for extraction and a Hypersil gold column was used for separation.

A method for doxorubicin quantification in HeLa cells and HT29 cells was developed by Kato et al. \textsuperscript{85} The extraction was done using LLE. Separation was done using a Capcell Pak C18 column maintained at 25°C. The isocratic mobile phase was composed of a 65:27 mixture of 50 mM sodium phosphate buffer and acetonitrile. The detector used was a fluorescence detector with an excitation wavelength of 470 nm and an emission wavelength of 590 nm.\textsuperscript{86} Another method for doxorubicin quantification in human leukemia cell was developed by Xu et al. where the LLE technique is used for extraction and a Hypersil gold column was used for separation. The mobile phase was in isocratic elution consisted of water (having 0.1% formic acid) and acetonitrile in 75:25 ratio. The mass ion source was ESI in positive mode and MRM was done using a triple quadrupole mass analyzer.\textsuperscript{87}

4-7 Cells

The drugs in which plasma concentration is not well correlated with its efficacy or toxicity, an analysis in their target cells of action is needed. But it may not be always possible to analyze the targeted tissue level of drug in an animal model. Therefore, a cell line is the best alternative to the drug needed to analyze in a specific target. Another advantage of choosing a cell line as a matrix is that it can easily be modified genetically or epigenetically which is very difficult in a live animal. Cell line study is widely used to identify the gene, cellular pathways and the biological mechanism involved in cancer pathogenesis.\textsuperscript{88}

For asulacrine, Zang et al. developed a method for MCF7 cells. The method used for extraction was PPT and the separation was achieved using a ZorbaxC18 column. The column temperature was set at 40°C. For separation, the mobile phase used was a mixture of water (having 2 mmol/L ammonium acetate and 0.1% acetic acid) and acetonitrile. For mass analysis, the source for ionization was ESI in positive mode and it was monitored in SRM mode with a triple quadrupole analyzer. This method was found to be helpful for finding out the subcellular concentration of asulacrine in MCF7 cells.\textsuperscript{89}

A method for doxorubicin quantification in HeLa cells and HT29 cells was developed by Kato et al. \textsuperscript{85} The extraction was done using LLE. Separation was done using a Capcell Pak C18 column maintained at 25°C. The isocratic mobile phase was composed of a 65:27 mixture of 50 mM sodium phosphate buffer and acetonitrile. The detector used was a fluorescence detector with an excitation wavelength of 470 nm and an emission wavelength of 590 nm.\textsuperscript{86} Another method for doxorubicin quantification in human leukemia cell was developed by Xu et al. where the LLE technique is used for extraction and a Hypersil gold column was used for separation. The mobile phase was in isocratic elution consisted of water (having 0.1% formic acid) and acetonitrile in 75:25 ratio. The mass ion source was ESI in positive mode and MRM was done using a triple quadrupole mass analyzer.\textsuperscript{87} Later, one more method for quantification of doxorubicin in MCF7 cells was developed by Ma et al. in which the extraction technique was PPT using methanol. Separation was achieved using a BEH C18 column set at room temperature. Gradient elution using the mobile phase acetonitrile and 0.1% formic acid in water was performed. Mass analysis was carried out using an ESI ion source in positive mode and quantification was performed in MRM mode.\textsuperscript{88}

Klawitter et al. developed a method for the quantification of imatinib in leukemia cells. The extraction technique used was PPT. The column used for separation was a Luna C18 maintained at 65°C. The mobile phase used was methanol and 5 mM ammonium acetate having 0.01% trifluoroacetic acid.

### Table 5: Bioanalytical methods for the analysis of anticancer drugs in tissues

| Compound     | Analytical technique | Extraction technique | Column, mobile phase, elution mode | Detection technique | Sample matrix | Quantification range | Reference |
|--------------|----------------------|----------------------|-----------------------------------|---------------------|---------------|----------------------|-----------|
| Alectinib    | LC-MS/MS             | PPT                  | BEH C18 column, HCOOH and ACN, gradient | Triple quadrupole in SRM mode | Mouse tissue homogenates | 2 - 2000 ng/mL | 72        |
| Apatinib     | LC-MS/MS             | PPT                  | Zorbax SB-C18 column, HCOOH in water and CH\textsubscript{3}OH, gradient | Triple quadrupole mass analyzer | Tissues of mice | 1 - 5000 ng/mL | 73        |
| AZD3759      | LC-MS/MS             | PPT                  | Hypersil GOLD C18 column, ACN and HCOOH in water, gradient | Triple quadrupole in SRM mode | Brain tissue homogenates | 1 - 1000 ng/mL | 74        |
| Bendamustine | LC-MS/MS             | PPT                  | Zorbax C18 column, water and CH\textsubscript{3}OH, gradient | Triple quadrupole in SRM mode | Mouse brain tissue homogenates | 5 - 2000 ng/mL | 75        |
| Brigatinib   | LC-MS/MS             | PPT                  | BEH C18 column, HCOOH and ACN, gradient | Triple quadrupole in SRM mode | Mouse tissue homogenates | 4 - 4000 ng/mL | 72        |
| Cabozantinib | UPLC-MS/MS           | LLE                  | UPLC BEH C18 column, ACN and water with HCOOH, gradient | Triple quadrupole in MRM mode | Tissues of rat | 5 - 5000 ng/mL | 75        |
| Imatinib     | HPLC                 | PPT                  | Inertil CN-3 column, water, CH\textsubscript{3}OH and TEA, isocratic | UV detection at 268 nm | ICR mouse tissue | 0.1 - 50 ng/mL | 77        |
| Lorlatinib   | LC-MS/MS             | LLE                  | C18 Waters column, HCOOH in water and CH\textsubscript{3}OH, gradient | Ion trap in SRM mode | Tissues of rat | 0.02 - 7.90 μg/mL | 78        |
| Panobinostat | UPLC-MS/MS           | LLE                  | UPLC BEH column, water and CH\textsubscript{3}OH, isocratic | Triple quadrupole in SRM mode | Mouse tissue homogenates | 2 - 2000 ng/mL | 72        |
| Pazopanib    | LC-MS/MS             | LLE                  | X Terra C18 column, water with HCOOH and ACN, isocratic | Triple quadrupole in MRM mode | Mouse tissue homogenates | 0.0025 - 0.8 μg/mL | 79        |
| Pomalidomide | LC-MS/MS             | PPT                  | Zorbax C18 column, water with HCOOH and ACN, gradient | Triple quadrupole in SRM mode | Mouse tissue homogenates | 3.9 - 1000 ng/mL | 80        |
| Sorafenib    | LC-MS/MS             | PPT                  | Polaris-3 C18 column, water with HCOOH and ACN, gradient | Triple quadrupole in MRM mode | Liver homogenates | 10 - 5000 ng/mL | 82        |
| Sunitinib    | LC-MS/MS             | LLE                  | Luna C8 column, ACN and CH\textsubscript{3}COONH\textsubscript{3}, isocratic | Triple quadrupole in MRM mode | Brain tissue | 4.12 - 1000 ng/mL | 83        |
| Ureidomustine| HPLC-DAD             | PPT                  | C18 column, NaH\textsubscript{2}PO\textsubscript{4}, and ACN, isocratic | Triple quadrupole in SRM mode | Brain tissue | 1.95 - 500 ng/mL | 84        |

For asulacrine, Zang et al. developed a method for MCF7 cells. The method used for extraction was PPT and the separation was achieved using a ZorbaxC18 column. The column temperature was set at 40°C. For separation, the mobile phase used was a mixture of water (having 2 mmol/L ammonium acetate and 0.1% acetic acid) and acetonitrile. For mass analysis, the source for ionization was ESI in positive mode and it was monitored in SRM mode with a triple quadrupole analyzer. This method was found to be helpful for finding out the subcellular concentration of asulacrine in MCF7 cells. A method for doxorubicin quantification in HeLa cells and HT29 cells was developed by Kato et al. The extraction was done using LLE. Separation was done using a Capcell Pak C18 column maintained at 25°C. The isocratic mobile phase was composed of a 65:27 mixture of 50 mM sodium phosphate buffer and acetonitrile. The detector used was a fluorescence detector with an excitation wavelength of 470 nm and an emission wavelength of 590 nm. Another method for doxorubicin quantification in human leukemia cell was developed by Xu et al. where the LLE technique is used for extraction and a Hypersil gold column was used for separation. The mobile phase was in isocratic elution consisted of water (having 0.1% formic acid) and acetonitrile in 75:25 ratio. The mass ion source was ESI in positive mode and MRM was done using a triple quadrupole mass analyzer. Later, one more method for quantification of doxorubicin in MCF7 cells was developed by Ma et al. in which the extraction technique was PPT using methanol. Separation was achieved using a BEH C18 column set at room temperature. Gradient elution using the mobile phase acetonitrile and 0.1% formic acid in water was performed. Mass analysis was carried out using an ESI ion source in positive mode and quantification was performed in MRM mode.
mixed in gradient elution mode. Total analysis time was 10.5 min. The mass spectrometer used a triple quadrupole mass analyzer with an ESI source in positive MRM mode. Daumar et al. developed an HPLC method for olaparib in SUM1315 breast cancer cell. PPT was employed as the sample extraction technique. The separation was performed using a Nova-Pak C18 column maintained at 35°C. The mobile phase was acetonitrile and ultra-pure water in a gradient elution mode. The detector used was DAD at 254 nm.

Wang et al. developed a method for quantification of paclitaxel in A549 cells where extraction was performed using the LLE technique and separation was achieved by using an Ascentis Express C18 column kept at 40°C. The mobile phase was acetonitrile and 0.1% formic acid in water in a ratio of 50:50. The triple quadrupole mass analyzer with positive ESI mode operated in MRM mode was used for analysis. Another method for paclitaxel quantification in A549 cell culture and cell lysate was developed by Baati et al. The method used for extraction was LLE and separation was performed using a Phenomenex Kinetex XB-C18 column. The mobile phase had a linear gradient of acetonitrile and 2 mM ammonium acetate containing 0.1% formic acid. The total chromatographic run time was 10 min. The mass analysis was performed in positive ion mode using ESI. Monitoring was done in MRM mode using a triple quadrupole mass analyzer.

Roche et al. developed a method for thalidomide quantification in lung cancer cell line using the LLE technique and a prodigy C18 column maintained at 20°C for separation. The premixed mobile phase having acetonitrile and water (3:7) containing 0.1% formic acid was employed. Total analysis time was 13 min. Mass analysis was performed in positive ESI mode using a triple quadrupole mass analyzer in MRM mode.

Table 6 summarizes the details of different bioanalytical method parameters reported for the analysis of anticancer drugs in various cell lines.

### 4-8 Rare biomatrices

There are various other types of rare matrices in which the concentration of drugs need to be monitored because of their typical distribution properties, site of action and metabolism characteristics. Analysis of drug in sweat or feces as a matrix is done when they are one of the routes of excretion. Analysis of a drug in CSF is done when the drug is targeting the brain or has the property to cross the BBB. Quantification of a drug in RLMs matrix may help to determine metabolic stability of the drug.

For gefitinib, Fang et al. developed a method for quantification in cerebrospinal fluid where extraction was performed using the SPE technique and separation was done using a Zorbax Eclipse C18 column. The temperature of the column was set at 40°C. The mobile phase used was 0.1% triethylamine solution and acetonitrile in gradient flow. DAD detector at 344 nm was employed. Total chromatographic run time was 10 min.

Wang et al. developed a method for quantification of ponatinib in rat feces using the LLE technique and separation was achieved using a Waters Cortecs column kept at 40°C. The mobile phase used was water (having 0.1% formic acid and 2 mM ammonium formate) and methanol in gradient elution. The mass detection was performed in MRM with ESI in positive ion mode.

Lankheetet al. developed a method for quantification of sunitinib in human sweat where extraction was performed using methanol. A Gemini C18 column kept at 40°C was used. The gradient mobile phase-A consisted of water having 10 mM ammonium hydroxide and phase-B consisted of methanol having 1 mM ammonium hydroxide, and the a total chromatographic run time was 10 min. Triple quadrupole mass analyzer is in MRM monitoring was carried out using ESI ion source in positive mode.

For quantification of vandetanib in CSF, Bai et al. developed a method using the LLE technique. A Kinetic C18 column was used with mobile phase consisting of a mixture of acetonitrile and ammonium formate (10 mM) in 50:50 ratio in isocratic elution mode for a total chromatographic run time of 3 min. The mass analysis was performed using a triple quadrupole mass analyzer in positive MRM mode with an ion spray interface.

Another method was developed by Amer et al. for vandetanib quantification in rat liver microsomes where extraction was performed using PPT and separation was achieved using a C18 column maintained at 22°C. The mobile phase was ammonium formate (10 mM) and acetonitrile (50:50) and total run time was 4 min. In mass spectrometry, ESI in positive ionization was employed and it was monitored in MRM mode with a triple quadrupole mass analyzer. Table 7 summarizes the details of different bioanalytical method parameters reported for the analysis of anticancer drugs in whole blood, synovial fluid, CSF, in vitro liver microsomes, feces and sweat. Figure 2 statistically describes the trend of the use of different extraction techniques for the preparation of biological samples.

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**Table 6** Bioanalytical methods for the analysis of anticancer drugs, cells

| Compound    | Analytical technique | Extraction technique | Column in mobile phase, elution mode | Detection technique | Sample matrix | Quantification range | Reference |
|-------------|----------------------|----------------------|--------------------------------------|---------------------|--------------|----------------------|-----------|
| Asulacrine  | LC/MS                | PPT                  | Zorbax C18 column, water and ACN, gradient | Triple quadrupole in SRM mode | MCF7 cells | 1 - 1000 ng/mL | 87        |
| Doxorubicin | UHPLC                | LLE                  | Capcell Pak C18 column in Sodium phosphate and ACN, isocratic | Fluorescence detector | HeLa and HT29 cells | 11.7 - 24.5 pg | 88        |
| LC-MS/MS    | LLE                  | Hyperis gold column, water with HCOOH and ACN, isocratic | Triple quadrupole in MRM mode | Human leukemia cells | MCF7 cells | 5 - 1000 ng/mL | 89        |
| LC-MS/MS    | PPT                  | BEH C18 column, water with HCOOH and ACN, gradient | Triple quadrupole in MRM mode | Human leukemia cells | MCF7 cells | 1 - 300 ng/mL | 90        |
| Imatinib    | LC/LC-MS/MS          | PPT                  | Luna C18 column, CH-CH and CHCOONH₂ gradient | Triple quadrupole in MRM mode | Human leukemia cells | 0.01 - 75 ng/mL | 55        |
| Olaparib    | HPLC                 | PPT                  | Nova-Pak C18 column, ACN and water, gradient | DAD at 254nm | SUM1315 cells | 200 - 2000 ng/mL | 91        |
| Paclitaxel  | LC-MS/MS             | LLE                  | Ascentis Express C18 column, ACN and HCOOH, isocratic | Triple quadrupole in MRM mode | A549 cells | 0.2 - 50 pmol | 92        |
| LC-MS/MS    | LLE                  | Phenomenex Kinetex XB-C18 column, ACN and ammonium acetate, gradient | Triple quadrupole in MRM mode | A549 cells | 2 - 600 pg/mL | 93        |
| Thalidomide | LC-MS/MS             | LLE                  | Prodigy C18 column, ACN and water with HCOOH, isocratic | Triple quadrupole in MRM mode | DLKP-A cells | 0.78 - 50 ng | 48        |

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For gefitinib, Fang et al. developed a method for quantification in cerebrospinal fluid where extraction was performed using the SPE technique and separation was done using a Zorbax Eclipse C18 column. The temperature of the column was set at 40°C. The mobile phase used was 0.1% triethylamine solution and acetonitrile in gradient flow. DAD detector at 344 nm was employed. Total chromatographic run time was 10 min.

Wang et al. developed a method for quantification of ponatinib in rat feces using the LLE technique and separation was achieved using a Waters Cortecs column kept at 40°C. The mobile phase used was water (having 0.1% formic acid and 2 mM ammonium formate) and methanol in gradient elution. The mass detection was performed in MRM with ESI in positive ion mode.

Lankheet et al. developed a method for quantification of sunitinib in human sweat where extraction was performed using methanol. A Gemini C18 column kept at 40°C was used. The gradient mobile phase-A consisted of water having 10 mM ammonium hydroxide and phase-B consisted of methanol having 1 mM ammonium hydroxide, and the a total chromatographic run time was 10 min. Triple quadrupole mass analyzer is in MRM monitoring was carried out using ESI ion source in positive mode.

For quantification of vandetanib in CSF, Bai et al. developed a method using the LLE technique. A Kinetic C18 column was used with mobile phase consisting of a mixture of acetonitrile and ammonium formate (10 mM) in 50:50 ratio in isocratic elution mode for a total chromatographic run time of 3 min. The mass analysis was performed using a triple quadrupole mass analyzer in positive MRM mode with an ion spray interface.

Another method was developed by Amer et al. for vandetanib quantification in rat liver microsomes where extraction was performed using PPT and separation was achieved using a C18 column maintained at 22°C. The mobile phase was ammonium formate (10 mM) and acetonitrile (50:50) and total run time was 4 min. In mass spectrometry, ESI in positive ionization was employed and it was monitored in MRM mode with a triple quadrupole mass analyzer. Table 7 summarizes the details of different bioanalytical method parameters reported for the analysis of anticancer drugs in whole blood, synovial fluid, CSF, in vitro liver microsomes, feces and sweat. Figure 2 statistically describes the trend of the use of different extraction techniques for the preparation of biological samples.
Current Scenario and Advancement in Analytical Methodology

Selection of biological matrices as a bioanalytical sample is carried out based on the site of action, mechanism of action, pharmacokinetics and intended use of the drugs. If the excretion of a drug and its metabolites is through urine, then analysis of the drug or its metabolites in urine is required. If the drug is meant to target any specific organ, then its quantification in that organ will provide its actual tissue distribution profile. If the site of action is inside the nucleus e.g. topoisomerase II inhibitors, then an intracellular study is performed to find the amount of the drug reaching the DNA. Sometimes, quantification in biomatrices like sweat, saliva, CSF, etc. is also performed based on different criterions. Some drugs require a highly sensitive technique to be detected and quantified with suitable accuracy and precision.

The advanced analytical techniques used for the determination of anticancer drugs in biological samples include LC-MS/MS, GC-MS, LC-UV, and CE-UV. Agents having UV characteristics are easy to be determined by UV detector in HPLC, although it does not provide high sensitivity. The level of detection through UV detection may not be satisfactory to determine the drug levels specifically in matrices like cells, tissues or PBMC. The level of detection is extremely low for the analyte in such type of matrices. Fluorescence spectroscopy may be an option for drugs having a fluorescent group like bendamustine (Table 2). In addition, sometimes other techniques like DPV or NACE are used for the detection of drugs in biomatrices.

To date, MS has been found to be the best technique for detection of analytes where biological samples are involved. MS offers several advantages for drug metabolite detection and pharmacokinetics of the drug in different biomatrices. One advantage of the MS technique is high sensitivity at the picogram level, which is particularly useful for quantification of an analyte in several rare biomatrices like CSF, PBMC, cells, etc. Nowadays, detection of drugs using LC-MS/MS has increased to around 80% for the analysis of drugs of all categories. Additionally, the MS technique is highly specific as it detects a particular mass transition for an analyte. Therefore, interferences from endogenous components become negligible. Recently, advancements in the detection technique based on mass technique offers much higher sensitivity compared to triple quadrupole mass analyzer.

Sample preparation also affects the sensitivity of an analytical method. Usually, the traditional sample preparation techniques like PPT, LLE and SPE are used. There are different advanced sample preparation techniques, which offer additional advantages in improving sensitivity and specificity in the analysis of anticancer drugs. Matrix solid phase dispersion reported to be helpful in extracting the analyte from highly viscous, solid and semi-solid biological samples. SCFE is a green sample extraction technique, which involves carbon dioxide, which provides higher recovery and better selectivity. DBS technique minimizes the requirement of sample volume as it uses a very low quantity of blood to quantify the analyte. However, there is still a requirement of complete automated, fast, reliable and sensitive sample extraction technique, which can help to recover a much higher amount of analyte from the biological samples.

5 Conclusions

Conventionally, HPLC with UV or fluorescence detector is used for the quantitative bioanalysis of the majority of the anticancer drugs. However, the limitation of this technique includes lower

![Summary of sample treatment procedure of anticancer drugs in different biomatrices](image-url)
sensitivity of the instrument compared to the modern analytical techniques. As in several steps of drug development, it is required to detect and quantitate the drugs or their metabolites in picogram or even much lower levels, tandem mass spectrometric techniques are becoming highly useful. Nowadays, the hyphenated LC-MS/MS technique is considered a useful alternative and is used in most of the current research for quantification of drugs in biological samples. A majority of the methods developed and published in the last few years employed LC-MS techniques for bioanalysis of anticancer drugs. The development of newer MS technology made it possible to use the bioanalytical methods to quantify the analytes at trace levels. The advanced modern type of mass analyzers offers much higher sensitivity compared to a triple quadrupole mass analyzer. Additionally, advanced sample extraction methodologies are allowing for faster and more specific extraction of the intended analytes. Advancement in the sample preparation technique is contributing equally to improve the sensitivity and selectivity of bioanalytical methods. In this article, bioanalytical aspects of anticancer drugs in different biological matrices have been discussed and summarized to give direction to the researcher for choosing appropriate techniques for quantification needs.

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8 Conflict of Interest

We do not have any conflict of interest to declare.

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