REVIEW

Application of salt-assisted liquid-liquid extraction in bioanalytical methods

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Abstract

This review provides a comprehensive analysis of bioanalytical methods employed for the quantification of drug molecules in various biological matrices, including human plasma, urine, breast milk, and mouse plasma. The study not only examines traditional sample preparation techniques such as protein precipitation (PP), liquid-liquid extraction (LLE), and solid-phase extraction (SPE), but also delves into the relatively new and innovative salting-assisted liquid-liquid extraction (SALLE). It offers a thorough comparison of analytical methods utilizing SALLE, focusing on key parameters such as analysis time, calibration range, and the type and quantity of salts and organic solvents used. This review aims to serve as an essential resource for researchers and practitioners in selecting the most suitable bioanalytical methods for pharmacokinetic studies and drug monitoring, ultimately enhancing data quality and analytical efficiency in both clinical and research settings.

Keywords: bioanalytical methods, chromatography, mass spectrometry, SALLE

Introduction

Bioanalytical methods are analytical methods that have the purpose of establishing the appropriate dosing schedule by considering the pharmacokinetic variability between individuals, performing routine drug analysis, detecting the abuse of drugs, and evaluating pharmacokinetics in new drug discovery. Since analyte concentrations are very low, the method must meet guidelines for reproducibility, accuracy, selectivity, and precision (Nováková & Vlčková, 2009). A bioanalytical method consists of 3 parts. These are the collection of the sample (serum, hair, saliva, blood, organ tissue, plasma, urine, feces), sample preparation, which is a crucial step to improve the performance of the method and minimize the matrix effect, and sample analysis (Moein et al., 2017). Bioanalyses are considered quite complex and difficult, because biological matrices are highly complex and the amount of analyte is quite low (Nazario et al., 2017). Sample preparation is an essential step to isolate the analyte from the matrix, minimize the matrix effect, and enrich the analytes if necessary (Li et al., 2019). It has a significant impact on data quality, analysis throughput, and employee satisfaction (Chang et al., 2007). This step should have the following goals: minimize or eliminate the consumption of hazardous and toxic organic solvents, reduce sample volume, reduce extraction time and increase yield, and should be easily implementable (Kabir et al., 2017). It is widely recognized that converting a sample into a format compatible with analytical instrumentation is the most time-consuming, labor-intensive, and error-prone step. This conversion can be as simple as dilution or filtration or can involve multi-step procedures (Clark et al., 2016; Theodoridis & Papadoyannis, 2006).

Sample Collection

Sample collection in bioanalyses has a critical role in maintaining the stability of the analyte in the sample matrix. This process involves the collection, processing, storage and transport of samples and often samples are collected from different facilities or laboratories and shipped worldwide (Pawula et al., 2013). Therefore, the handling and transport of samples needs to be meticulously planned and implemented. In bioanalysis, it is important that analyte concentrations in samples accurately reflect the concentrations at the time of collection. For this purpose, it may be necessary to use stabilizers to prevent degradation or non-specific binding of analytes (Hilhorst et al., 2015). In recent years, clinical laboratories and pharmaceutical manufacturers have shown a marked increase in interest for drug analysis in biological fluids. An increasing focus in clinical chemistry and forensic toxicology is the use of alternative samples. Sweat, hair and oral fluids are some of the non-traditional samples that have attracted attention. The collection of oral fluids from these samples does not require the same level of expertise as blood sampling, but physiological factors can still lead to fluid deficiencies. (Fura et al., 2003). Moreover, the intake of certain foods and the use of techniques that stimulate oral fluid production can influence drug concentrations. The clinical acceptance of monitoring drug levels in oral fluids is limited to a few drugs, as the correlation between plasma and oral fluid levels is not sufficiently strong (Elmongy & Abdel-Rehim, 2016). Pharmacokinetic and metabolism studies typically involve monitoring drug and metabolite concentrations in samples such as plasma, urine, or bile, which are stored, processed and analyzed after collection (Fura et al., 2003).

Conventional sample preparation techniques

Techniques such as liquid-liquid extraction (LLE), protein precipitation (PP), and solid phase extraction (SPE) were historically prevalent in sample preparation, but are now regarded as traditional methods. In recent years, there has been a swift advancement in innovative sample preparation techniques within the realm of bioanalysis (Kole et al., 2011).

Protein precipitation is a technique that uses water-miscible organic solvents to denature and agglutinate proteins, thereby removing analytes from plasma samples. In this process, proteins are usually precipitated by disrupting their hydrogen bonds with solvents such as acetonitrile or methanol, which helps to eliminate interferences from large molecules (Ma et al., 2008). The protein precipitation method is widely preferred due to its low cost, rapid results and allows rapid development of analyses, especially in preclinical pharmacokinetic studies of drug discovery (Ping et al., 2022). Furthermore, it provides sufficient purity for LC-MS analyses and, when the number of samples is large, the process can be more efficient and automated, with the use of 96-well plate structure (Zhang et al., 2022). However, this method also has some negative aspects. Steps requiring manual handling may prevent full automation of the process and limit the capacity of the operator to perform other tasks in parallel (Ping et al., 2022). Furthermore, due to the sensitivity of proteins to different denaturation conditions, complete precipitation of some proteins may be difficult, which may prevent complete separation of analytes with unwanted proteins (Nfor et al., 2011). Therefore, although the protein precipitation method is practical in the laboratory environment, it has some limitations, especially in large-scale and high-sensitivity studies.

LLE is a commonly utilized clean-up method for extracting compounds from aqueous samples. This technique facilitates the transfer of target compounds between phases by bringing immiscible or only partially soluble liquids into contact. Moreover, LLE is recognized as one of the most traditional and extensively applied methods in sample preparation for both qualitative and quantitative analysis, making it a preferred choice for sample pretreatment in various analytical methods. (Cantwell & Losier, 2002; Lorenzo-Parodi et al., 2023). The main advantages of this method include its simplicity, low costs, and ease of scale-up (Mazzola et al., 2008). LLE also exhibits several limitations. The process demands significant quantities of organic solvents, which often possess toxic characteristics and present environmental and health hazards. Moreover, it is recognized as a process that requires considerable time and effort from the analyst, thereby increasing the likelihood of human error (Lorenzo-Parodi et al., 2023). To overcome these disadvantages, different strategies have been proposed and utilized. These strategies aim to minimize the occurrence of errors by reducing solution consumption, minimizing waste generation, and limiting operator intervention and exposure (Silvestre et al., 2009). In conclusion, although liquid-liquid extraction is a common and effective cleaning technique, more sustainable alternatives need to be developed, considering the environmental and operational challenges.

SPE is a common method employed for sample preparation aimed at isolating specific analytes, typically from a mobile phase. During this process, analytes are captured in a solid phase and stay there for the duration of the sampling. These analytes are then retrieved from the solid phase through thermal desorption into a liquid phase. The primary objectives of SPE include enhancing the concentration, purifying the sample, and moving analytes from the original sample matrix to an alternative solvent phase (Poole, 2003). SPE has become a popular sample preparation method of choice in many applications, because it offers many advantages over other conventional methods. The technique has been developed as an alternative that eliminates the use of large quantities of solvents, long processing times and procedural steps, as well as potential sources of error. Furthermore, SPE can be used in combination with other analytical methods and sample preparation techniques (Ötles & Kartal, 2016). However, SPE also has some disadvantages, including the difficulty of mastering the technique, the perceived complexity of the method due to the large number of options for changing solvent and pH conditions, the need for additional time

due to the need for several steps, and the generally higher cost compared to simple LLE (Płotka-Wasylka et al., 2015).

Salting-assisted liquid-liquid extraction

Salting consists of incorporating an electrolyte into an aqueous solution to improve the solute's distribution (Sazali et al., 2019). Salting-assisted liquid-liquid extraction (SALLE) utilizes the principle of separating water-miscible organic solvents from aqueous biological fluids, such as plasma, to facilitate the extraction of a diverse array of drugs and metabolites, including various hydrophilic compounds. The resulting organic phase can be employed directly for bioanalysis or may require minimal dilution. SALLE offers a more straightforward approach compared to protein precipitation and yields cleaner extracts due to effective phase separation. Moreover, SALLE is noted for being quicker, more eco-friendly, and cost effective compared to traditional liquid-liquid extraction (LLE) and solid-phase extraction (SPE). In the SALLE process, incorporating inorganic salts like sodium chloride, sodium sulfate, or magnesium sulfate into the mixture induces the separation of organic solvents such as methanol, isopropanol, acetone, ethanol, and acetonitrile, creating a two-phase system (Martins et al., 2024; Tang & Weng, 2013). When these salts are used, the salting-out effect boosts the solution's ionic strength, reducing the solubility of weak electrolytes in water. This process enhances the extraction of target analytes into the organic phase, achieving high efficiency for extracting polar or moderately polar compounds from aqueous samples. Additionally, SALLE methods ensure compatibility of extraction solvents with most analytical instruments, particularly chromatographic systems, allowing direct injection of the extract for subsequent analysis (Bekele et al., 2023).

Bioanalysis

Bioanalytical techniques have undergone significant advancements over the years. Previously, chromatography HPLC-UV detection was regarded as the gold standard, but recently it has been largely replaced by tandem mass spectrometry (MS/MS)(Nováková, 2013). Today, mass spectrometry is considered the gold standard for bioanalytical studies, as it is a highly sensitive and specific method (Douxfils et al., 2016). Although LC-MS/MS has been recognized as a powerful method of analyses, it has disadvantages such as matrix effect, ion suppression or enhancement, and interferences from metabolites (Matuszewski et al., 2003). GC-MS is one of the most widely used analytical techniques for identifying and quantifying substances in complex matrices. It has many applications in environmental science, forensics, healthcare, medical and biological research (Sparkman et al., 2011). Volatile and low molecular weight analytes can be analyzed directly in GC-MS, but if the analyte contains polar functional groups oris not thermally stable at the temperatures required for separation, or non-volatile, derivatization must be performed before analysis (Koek et al., 2011).

Table 1. The methods presented in the literature.

References	Instrument	Matrix	Calibration Range	LLOQ	Analysis Time	Salt type and amount	Organic solvent type and amount
(Gupta et al., 2010)	HPLC-UV	Plasma	amoxapine: 0.0025–15 mg/L nortriptyline: 0.0025–15 mg/L	0.0025 mg/L	12 min	2.5 g of ammonium sulphate	0.5 mL acetonitrile

(Kul & Sagirli, 2023b)	GC-MS	Plasma	Biperiden: 0.5 and 15 ng/mL	0.5 ng/mL	8 min	200 mg NaCl	300 µl acetone
(Pourhossein & Alizadeh, 2018)	HPLC-UV	Plasma	Carvedilol: 5-500 µg/L	3.3 µg/L	12 min	1.5 gr NaCl	1.5 ml acetonitrile
(Al et al., 2024)	LC-MS/MS	Plasma	Haloperidol: 1-15 ng/ml	1 ng/ml	4 min	200 mg NaCl	300 µl acetonitrile
(Hammad et al., 2021)	HPLC-UV	Plasma	Alogliptin: 0.1–50 µg/mL	0.06 µg/mL	8 min	250 mg NaCl	500 µL acetonitrile
(Manousi et al., 2022)	HPLC-DAD	Urine	Piroxicam-Meloxicam: 0.1–4.0 µg/mL	0.1 µg/mL	15 min	480 µL (2.5 M) of sodium sulfate	600 µL acetonitrile
(Myasein et al., 2009)	LC-MS/MS	Plasma	Lopinavir: 19.2-16 ng/mL ritonavir: 9.73-8110 ng/mL	Lopinavir: 20 ng/mL ritonavir: 10ng/mL	0.40 min	100 L of 3 M zinc sulfate	200 µL of acetonitrile
(Zhang et al., 2010)	LC-MS/MS	Plasma	“simvastatin simvastatin acid”0.097-51.1 ng/mL	0.094 ng/mL	2 min	50 µL o5 M ammonium formate buffer	200 µL of acetonitrile
(Xiong & Yang, 2015)	LC-MS/MS	rat plasma	trimetazidine 0.1–100 ng/mL	0.1 ng/mL	2.5 min	25 µL of 5 M ammonium formate	100 µL of acetonitrile
(Kvamsøe et al., 2020)	LC-MS/MS	blood	Tacrolimus: 0.4–85 ng/mL Sirolimus: 1.4–84 ng/mL Everolimus: 0.06–83 ng/mL Cyclosporine: 0.4–959 ng/mL	Tacrolimus: 0,4 ng/mL Sirolimus: 1,4 ng/mL Everolimus: 0,06 ng/mL Cyclosporine: 0.4 ng/mL	1.1 min	100 µL of 5M NaCl	350 µL acetonitrile
(Alshishani et al., 2017)	HPLC-UV	plasma urine	metformin: 20–2000 µg/L buformin: 20–2000 µg/L phenformin: 20–2000 µg/L	metformin: 13 µg/L buformin: 12 µg/L phenformin: 17 µg/L	16 min	0.48 mg NaOH	400 µL acetonitrile
(Hajkova et al., 2016)	LC-MS	tissue samples	methoxetamine 2.5–250 ng/g	2.5- 5 ng/g	8 min	200 µL 10 mmol/L NH4HCO3	100 µL acetonitrile
(Sparidans et al., 2016)	LC-MS/MS	mouse plasma	afatinib 0.5–500 ng/ml	0.5 ng/ml	1.2 min	5 µl of 3 M magnesium chloride	25 µL acetonitrile

(Kul & Sagirli, 2023a)	LC–MS/MS	breast milk	cefuroxime 25-1000ng/ml	25 ng/ml	8 min	200 mg zinc sulfate	300 µl acetonitrile
(Yang et al., 2015)	LC–MS/MS	plasma	atorvastatin 0.0200–15.0 ng/mL	0.0200 ng/ml	2.2 min	100 µL of 6 M ammonium acetate	400 µL acetonitrile
(Stratigou et al., 2020)	HPLC-FL	Urine	doxorubicin 100-2000 ng/ml	100 ng/ml	15 min	1 mL of Na2SO4 solution 2.35 mol/L	2.35 mL acetonitrile
(Zhao et al., 2020)	LC-MS	plasma	entecavir 0.05-20 ng/mL	0.05 ng/mL	11 min	500 µl of MgSO4 solution (37.5%)	1 mL acetonitrile

Conclusions

SALLE offers many advantages over conventional methods. SALLE uses the principle of separation of water-immiscible organic solvents to extract drugs and metabolites from biological fluids with high yields. This method offers clean extracts and considered an eco-friendly approach compared to PP and LLE. In addition, SALLE is faster and cost effective, making analytical processes more efficient. The results obtained from the studies show that the use of the SALLE technique in combination with different analytical methods provides high performance in biological samples. In particular, LC-MS/MS studies using salts such as ammonium sulphate, magnesium sulphate and zinc sulphate provided faster analysis times and lower LLOQ values. This demonstrates that the integration of the SALLE technique with various analyzers increases the capacity for fast and sensitive measurements. However, the applicability and efficiency of each method varies depending on factors, such as the compound being analyzed, biological matrix, instrument and analysis time. Shorter analysis times may be preferable, especially for laboratories working with high sample numbers, while longer analysis times generally offer higher precision and accuracy. In conclusion, the flexibility and versatility of the SALLE method increases its adaptability to different analytical needs, making it a promising alternative for various bioanalytical applications.

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Conflict of interest

The authors declare no conflict of interest.

Data availability statement

Data sharing is not applicable to this review article as no datasets were generated or analyzed during the current study.

Ethics committee approval

Ethics committee approval is not required for this study.

Authors' contribution statement

Study conception and design: SA, OS; **Data collection**: SA; **Manuscript draft preparation**: SA **Supervisor:** OS. All authors reviewed the results and approved the final version of the manuscript.

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