DRUG ANALYTICAL RESEARCH
ISSN: 2527-2616 *www.seer.ufrgs.br/dar*

Valproic acid extraction methods in human samples using gas chromatography: a review

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Valproic acid (VPA) is widely used as an important drug for the treatment of epilepsy, seizures, bipolar disorders, and migraines. However, its narrow therapeutic range lead to risks of unintentional poisoning and potential suicide. Symptoms of VPA intoxication include drowsiness, hyperactivity, confusion, ataxia, coma, and sometimes can mimic brain death. Severe cases necessitate intensive care observation and evaluation of blood/plasma concentrations to determine the severity of poisoning. Analytical methods are crucial for monitoring drugs in biological fluids. This review aimed to describe the extraction methods used for the analysis of VPA in human biological samples using gas chromatography. Among 19 studies reviewed, liquid-liquid extraction (LLE), liquid phase microextraction (LPME), and solid-phase microextraction (SPME) are the most used extraction methods. Considering the laborious nature and large sample volumes required for LLE and solid-phase extraction (SPE), more efficient microextraction methods are needed. LPME, dispersive liquid-liquid microextraction (DLLME), air-assisted liquid-liquid microextraction (AALLME), and others offer promising alternatives. LPME, in particular, is favored ecologically due to reduced solvent usage compared to LLE. Novel approaches like dried blood spots (DBS) present emerging options for sample collection, helping in analytical and therapeutic monitoring contexts. Selection of extraction protocol hinges on method objectives, sample type, and laboratory capacity.

Keywords: Valproic acid, extraction methods, gas chromatography, sample preparation.

Article received at 03/04/2024 and accepted at 04/09/2024.
https://doi.org/10.22456/2527-2616.138963

Introduction

In clinical toxicology, an accurate diagnosis is a crucial step in poisoning management. In this way, toxicological analysis proves to be an important tool in this context. This analytical approach comprises several key steps, starting with screening and identification of potentially toxic compounds, followed by the quantification of relevant substances and the interpretation of the obtained results (1).

Valproic acid (VPA) has been used as an antiepileptic drug since 1978 and was later approved by the US FDA (Food and Drug Administration) for bipolar disorder treatment in 1995. Available in salt form, sodium valproate, it is widely prescribed as a monotherapy treatment globally. After administration, it is rapidly absorbed from the gastrointestinal tract, with peak plasma levels achieved within 1 to 4 hours (2-3). VPA (**Figure 1**) is a C8-branched carboxylic acid composed of two propyl groups and an acetic acid moiety, belonging to the group of short-chain fatty acids, also known as 2-propylpentanoic acid (4-6).

VPA is widely used in treating epilepsy, bipolar disorder, and migraine headache prevention. However, its use may lead to adverse effects such as hepatotoxicity. Acting as a central nervous system depressant, VPA has been associated to overdose cases, including intentional exposure for suicidal purposes. Toxic doses of VPA can lead to various clinical manifestations, including somnolence, hyperactivity, confusion, ataxia, coma, cardiac effects, parkinsonism, thrombocytopenia, platelet dysfunction, and aplastic anemia. Ingestion of VPA can mimic brain death with the absence of brain stem reflexes and pupillary responses to light (7-9). Intensive monitoring of patients may be necessary depending on the severity of poisoning, and the measured blood/plasma concentration can help predict the extent of the poisoning. In some cases, treatment for life-threatening poisonings may involve the administration of activated charcoal, hemoperfusion, or hemodialysis (1-2).

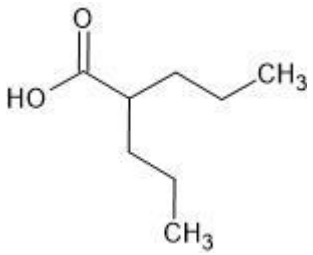

Figure 1. Chemical structure of valproic acid.

Monitoring VPA levels in patient plasma is crucial, especially during dose adjustment, concomitant medication use, or changes in the patient's clinical condition (7-9). Long-term or permanent use requires close monitoring of blood concentration, particularly in

children and the elderly, due to its proximity to toxic levels (2). In treating epilepsy, therapeutic concentrations of VPA typically fall within the range of 50-100 $\mu g.mL^{-1}$ (10-12). During controlled therapy, the therapeutic serum/plasma concentration of VPA is between 20-100 $\mu g.mL^{-1}$, while concentrations exceeding 120-150 $\mu g.mL^{-1}$ are considered toxic, when serum concentrations exceed 200 $\mu g.mL^{-1}$ the toxic effects become more frequent (7, 12).

The majority of VPA is metabolized, with less than 4% excreted unchanged in urine (11). The metabolism of VPA is complex and remains under investigation. Established pathways of VPA metabolism consist of β-oxidation in the tricarboxylic acid cycle (acetylation), oxidation involving cytochrome P-450 isoenzymes (P-oxidation), and glucuronidation. Certain metabolites exhibit anticonvulsant properties, while others are considered toxic and can affect various systems. Common effects of toxic metabolites of VPA include hepatotoxicity, teratogenicity, dermatitis, alopecia, thrombocytopenia, and central nervous system depression (11, 27).

VPA is a widely tested drug for therapeutic drug monitoring (TDM), which involves measuring specific drugs at intervals to maintain a constant drug concentration in the bloodstream. TDM is crucial due to individual variations in drug absorption, metabolism, and potential interactions with other medications. Drugs like antiepileptics, psychiatric drugs, cardiac drugs, antibiotics, bronchodilators, immunosuppressants, anti-cancer agents, and protease inhibitors commonly require TDM.

To monitor drugs effectively in human body fluids, accurate, sensitive, simple, rapid, and cost-effective analytical methods are essential. Immunoassays are commonly utilized in TDM laboratories due to their ease of use. However, it does have limitations in terms of selectivity, which can generate false positive and inaccurate results. Specifically, it is frequently employed to determine the free concentration of a drug in clinical TDM, but it is limited to detecting a single drug and may be susceptible to cross-reactions with their metabolites or other substances. This limitation may affect its specificity for detecting VPA, particularly in individuals with the use of different psychoactive drugs (13-14).

Gas chromatography (GC), liquid chromatography (LC), and capillary electrophoresis are frequently employed techniques for the quantification of VPA in biological matrices. The volatile nature of VPA often leads to a preference for GC-MS method, which provides greater resolution and enhances the sensitivity of the assay (4, 8). Gas chromatography is easy to operate and cost-effective, making it a promising candidate for widespread application as a conventional assay in clinical routine therapeutic drug monitoring. Recently, GC–MS/MS has gained popularity for its highly sensitive, selectable, and rapid multiple reaction monitoring capabilities, enabling high-throughput analysis of biological samples (3, 13, 23). Therefore, the objective of this study is to summarize the extraction methods found for the analysis of VPA in human biological samples using GC in recent years.

Methodology

A literature review was conducted with a focus on extraction methods of VPA in human samples using GC. A comprehensive search in the Pubmed database was carried out, using the keywords "valproic acid gas chromatography", giving a result of 354 articles. Then, published articles covering the period from 2001 to 2022 were filtered. The inclusion criteria were articles in the English language that addressed the analysis of VPA in human biological samples using gas chromatography. Articles that did not use human samples, did not explain the method, or were in other languages were excluded from the review. **Figure 2** provides an organizational chart showing the number of articles initially found and the included and excluded articles based on the specified criteria. In total, nineteen relevant studies were selected.

Of the nineteen studies found on the analysis and development of analytical methods for VPA in human samples using GC: eleven articles used GC-MS, two articles used GC-MS/MS, and six articles used GC-FID. Instrumental chromatographic methods, matrix used and extraction methods are summarized in **Table 1**.

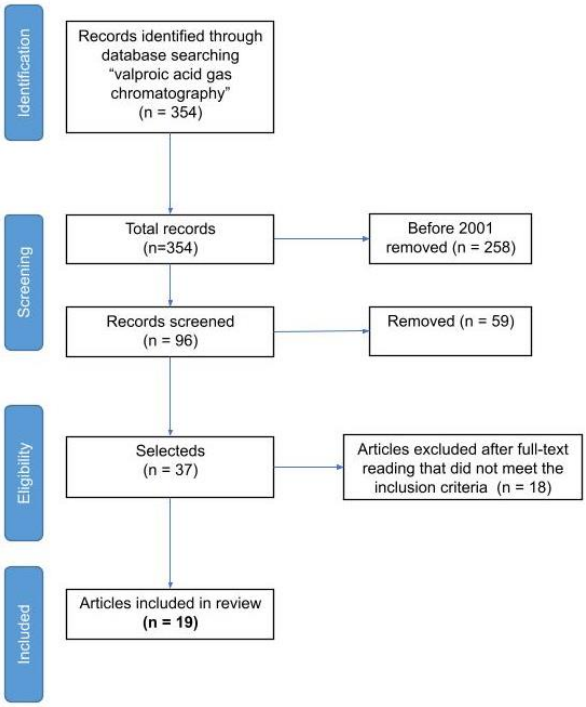

Figure 2. Flow chart of included and excluded articles in this review.

Table 1. Summary of instrumental chromatographic analysis described for valproic acid.

Extraction	Method	Chromatographic conditions	Matrix	Derivatization	Linear range ($\mu g.mL^{-1}$)	Ref.
LLE	GC-MS	HP-1MS fused silica capillary column (12 m x 0.25 mm i.d. x 0.25 mm), helium at 1mL/min, inlet temperature: 280ºC, oven temperature: initial of 85ºC and raised by 35ºC/min to 310ºC, splitless mode	plasma	-	20-200	1
LLE	GC-MS	column DB-5MS (15m x 0.25mm i.d.), split ratio 10:1 at 260ºC, helium at 1,5 mL/min, initial temperature 50ºC increase of 40º C/min to 300ºC	plasma	MSTFA	0.47-120	5
LLE	GC-FID	DB-FFAP (30 m × 530 µm×1 µm) maintained at 155 °C, injection port: 220ºC, FID: 250 °C, split ratio 1:1	plasma	-	0.56-28.11	13
LLE	GC-MS	column DB-5ms, 30 m, 0.25 µm i.d., starting temperature 100ºC increased by 20ºC/min to 280ºC, helium at flow rate 1.5 mL/min	blood	-	NR[1]	16
LLE	GC-MS	HP-1MS fused silica capillary column (30 m × 0.25 mm × 0.25 µm), oven temperature at 50 °C for 2 min, and then increased from 50 °C to 150 °C at a ramp of 15 °C /min, and from 150 °C to 280 °C at a ramp of 40 °C /min, injection port: 250 °C, detector: 280 °C, split ratio of 1:10	blood and urine	TMSDM	1.0-250	17
SPE	GC-MS	TraceGold TG-5MS column (30 m × 0.25 mm i.d. × 0.25 µm), initial temperature of 80°C, then increased 50°C/min up to 140°C with a final ramp of 30°C/min up to 290°C	blood	trimethyl ammonium hydroxide:DMSO	20-120	19
LPME	GC-FID	BP-10 14% cyanopropylphenyl dimethylsiloxane fused-silica capillary column (25 m × 0.22 mm I.D., 2.5µm film), injection port: 250ºC, detector: 280ºC, split ratio 20:1, oven temperature from 80 ◦C to 140 ◦C at a rate of 15 ◦C/min then, the temperature was raised 40 ◦C/min to 280ºC	serum	-	2-20	8
DLLME	GC-FID	HP-5 capillary column (30 m × 0.32 mm i.d., 0.25 µm), injection port: 270 °C in the splitless mode, oven temperature: initial 80 °C to 140 °C at a rate of 15 °C/min, then raised at 40 °C/min to 250 °C, FID temperature: 280 °C	plasma	-	6-140	7
DLLME	GC-MS/MS	TG-5MS capillary column (5% phenyl–95% dimethylpolysiloxane; 30 m × 0.25 mm id × 0.25 µm), initial oven temperature: 60°C increased up to 150°C at the rate of 10°C/min, further increased to 280°C at the rate of 40°C/min, helium at 1mL/min, splitless mode	urine	MCF	0.005-0.01	9
AALLME	GC-FID	HP-5 capillary column (30 m × 0.32 mm i.d. x 0.25 µm), temperature of the oven: 70 °C increased at 15°C/min to 200°C, and then increased at 20°C/min 300°C, temperature of FID: 300°C	plasma	-	0.2-100	11
SPME	GC-MS	HP-5MS fused-silica capillary column (30 m x 0.25 mm i.d. x 0.25 µm film), helium at 1mL/min, injector temperature at 250ºC, column temperature program: initial 40ºC, increase to 300ºC at 10ºC/min	plasma	pyridine and isobutyl chloroformate	2-100	3

SPME	GC-FID	J&W DB-WAX (polyethylene glycol) capillary column (30m x 0,25 mm i.d., 0,25 µm), initial temperature 80ºC raised at 20ºC/min to 240ºC, injection port: 150ºC and detector: 250ºC, splitless mode	serum	-	0.25-100	14
SPME	GC-FID	J&W DB-WAX (polyethylene glycol) capillary column (30 m x 0.25 mm i.d., film 0.25 mm). Initial temperature 80ºC and held for 1 min, raised by 20ºC/min to 240ºC and held for 5 min, injection port and detector: 250ºC, splitless mode	serum	-	0.20-100	22
BioSPME	GC-MS	Rtx-WAX column (30 m × 0.25 mm × 0.25 µm), injector temperature was 240 ◦C, split 1:40, helium at 1,5 mL/min, initial column temperature: 80 ◦C increased by 25 ◦C/min until 240 ◦C	serum	-	10-150	10
QuEChERS	GC-MS/MS	Rxi-5Sil MS (30 m × 0.25 mm i.d.; 0.25µm), initial temperature was held at 60 °C for 2 min, increased to 320 °C at 20 °C/min, helium at 1mL/min, splitless mode	plasma	-	0.05-5	23
SALLE	GC-MS	HP-5MS UI column (30 m × 0.25 mm, 0.25 µm), helium at 1mL/min, injector: 250 ◦C, source: 280 ◦C, starting oven temperature was 80 ◦C (1.2 min held) and it increased at a rate of 25 ◦C/min to 250 ◦C, split mode 10:1	plasma	esterification with methanol	0.075-15.0	4

[1]NR: not reported

Results and Discussion

Extraction Methods

Traditional sample pretreatment techniques, such as solid phase extraction (SPE) and liquid-liquid extraction (LLE) are known for their time-consuming. LLE also involves the use of large volumes of toxic solvents. In contrast, SPE is a highly selective sample preparation technique that isolates the target analyte using a solid sorbent. However, SPE has its drawbacks, such as potential variability in packings, irreversible adsorption of some analytes on cartridges, and more complex method development (7).

In the evaluated studies, five used LLE (1, 5, 13, 16, 17), and one used SPE (19) for VPA extraction. Due to the laborious procedures and need for large volumes of samples needed in LLE and SPE, it becomes essential to replace these extraction methods with more efficient microextraction sample preparation methodologies (9, 11). In this review, other extraction methods were also studied, such as liquid phase microextraction (LPME) (8), dispersive liquid-liquid microextraction (DLLME) (7, 9), air-assisted liquid-liquid microextraction (AALLME) (11), solid-phase microextraction (SPME) (3, 14, 22), BioSPME (10), QuEChERS (23) and salting-out liquid-liquid extraction (SALLE) (4). We also studied the extraction methods used in the analysis of VPA collected by Dried Blood Spots (2, 12, 24).

LLE

Liquid-liquid extraction involves extracting the analyte by adding a solvent that is immiscible with the sample and in which the analyte exhibits a higher affinity to form two distinct liquid phases, followed by phase separation and recovery of the analytes (15).

A rapid, simple, and cost-effective GC/MS method for the measurement of VPA, salicylic acid, paracetamol, phenobarbital, phenytoin, and primidone was developed by Meyer and colleagues (2011). The extraction process involved adding 50 µL of an internal standard (IS) solution (phenobarbital-d_5) and 450 µL of butyl acetate to 200 µL of blank human plasma samples. In emergency toxicology, this approach enables the simultaneous, rapid, and uncomplicated quantification of five relevant drugs in poisoning diagnostics. A notable advantage is the ability to detect all these compounds in a single GC run instead of utilizing individual immunoassays. The extraction efficiency achieved was 110±5% at low-quality control (QC) concentration and 105±3% at high QC concentration for VPA. The linearity ranges were established as 20–200 µg.mL^{-1} for VPA (1).

Leis *et al*. (2003) employed LLE for the extraction of valproate from acidified plasma with 500 µL of hexane. The analysis was carried out using gas chromatography coupled to mass spectrometry (GC-MS). The calibration curves exhibited linearity within a range of 0.47–120 µg.mL^{-1}, and the limit of quantification (LOQ) was established at 0.47 µg.mL^{-1}. A stable-isotope [$^{18}O_2$]VPA was synthesized for use as an internal standard (IS). Following extraction, the organic layer was dried and derivatized with N-methyl-N-trimethylsilyltrifluoro-acetamide (MSTFA), which included 1% trimethylchlorosilane in pyridine at a ratio of 2:1 (v/v) (5). The VPA derivatization process in GC may be required, due to the considerable polarity of the drug, in

addition to the possibility of thermal degradation (3, 9). For this, derivatization reactions, such as silylation, have been observed in different studies (3, 5, 9, 17, 19, 24). The study from Leis *et al*. (2003) showed a good extraction recovery, surpassing 90%. The developed method demonstrated effectiveness in batch analyzing over 800 plasma samples (5).

In 2020, Gu and colleagues developed a method for monitoring VPA in plasma samples using GC-FID. 100 µL of dichloromethane was employed for the extraction procedure. The obtained LLOQ was 0.56 $\mu g.mL^{-1}$ with a linear range of 0.56–28.11 $\mu g.mL^{-1}$. Given that individuals with epilepsy often use a combination of antiepileptic drugs, the researchers investigated potential interference from other drugs in plasma samples. Notably, common antiepileptic drugs such as carbamazepine, phenytoin, oxcarbazepine, and lamotrigine showed no interference with the chromatographic peak (13).

Koller *et al*. (2009) analyzed three heart blood samples from a *post-mortem* case using GC/MS. The samples were extracted, first with 500 µL of chloroform, followed by 1 mL of hydrochloric acid. Blood analysis revealed the presence of naftidrofuryl, disulfiram, VPA, and mefenamic acid. The VPA concentration was determined to be 50 $\mu g.mL^{-1}$, falling within therapeutic levels and significantly below lethal concentrations. Despite the VPA concentration being within the therapeutic range, a remarkable finding was the exceedingly high concentration of 7.5 $\mu g.mL^{-1}$ of naftidrofuryl in the whole blood. This elevated naftidrofuryl concentration was identified as the cause of death, leading to the conclusion that the most probable modus operandi of death was suicide. In this specific case, the quantification of VPA was essential to exclude it as the main cause of the poisoning that led to the death (16).

Namera *et al*. (2022) analyzed VPA in blood and urine using GC/MS, first 0.1 mL of sample (blood/urine) were mixed with 1 mL of hydrochloric acid in a 2 mL siliconized microtube. VPA was extracted in 0.5 mL of methyl tertiary-butyl ether (MTBE), and then derivatized using 10 µL of trimethylsilyldiazomethane (TMSDM) and 50 µL of methanol. The LOD found was 1,0 $\mu g.mL^{-1}$ for whole blood and urine, with a calibration range of 2,0 to 250 $\mu g.mL^{-1}$. The authors conducted a study to explore the use of 2-ethylhexanoic acid (2-EHA) as a cost-effective alternative internal standard. Deuterium-labeled products, like VPA-d_6, are expensive, and since 2-EHA has a similar structure to VPA, it was considered a potential substitute. The research revealed no significant differences in recoveries between VPA-d_6 and 2-EHA. As a result, 2-EHA was chosen as the internal standard for subsequent studies (17).

SPE

Solid-phase extraction (SPE) is a selective extraction method employing a packed solid sorbent (such as silica or polymer) to isolate the analyte. In this process, the aqueous sample is passed through a short column packed with an appropriate solid sorbent, where the solutes are adsorbed. Subsequently, organic solvents with high elution strength are employed to recover the analytes from the sorbent, resulting in their enrichment. As main advantages, the use of SPE provides excellent sample clean-up, sensitivity gain, minimizes solvent usage and waste generation. However, compared to other extractions, such as LLE, SPE cartridges have a high associated cost (7, 18). In this review, only one study used SPE for VPA analysis in biological samples.

Stephenson *et al*. (2016) introduced a SPE method to analyze VPA, salicylic acid, and ibuprofen for whole blood samples. In this work, butylated derivatives were generated to improve sensitivity in GC-MS analysis. The extraction process involved UCT Clean Screen® ZSTHC020 mixed-mode cartridges, which were preconditioned with methanol, deionized water, and phosphate buffer (pH 6). Derivatization was achieved by adding 100 µL of a trimethyl ammonium hydroxide solution:DMSO mixture (1:1, v/v), along with 50 µL of iodobutane. The study demonstrated a LOD and LOQ of 1 $\mu g.mL^{-1}$ for VPA, with a range linearity up to 120 $\mu g.mL^{-1}$. The authors indicated an extraction recovery rate of 10% for salicylic acid, 81% for ibuprofen, and 58% for VPA (19).

LPME

Liquid-phase microextraction (LPME) is a solvent-minimizing method in which only a few microliters of solvents are used, making it quick, cost-effective, and environmentally friendly with minimal exposure to toxic organic solvents. LPME integrates extraction, concentration, and sample introduction into a single step, offering advantages in simplicity, automation, and equipment requirements. LPME includes different modes, such as static LPME, dynamic LPME, and hollow-fiber liquid-phase microextraction (HF-LPME). One successful application of LPME is headspace liquid-phase microextraction (HS-LPME), which effectively extracts volatile to semi-volatile compounds without interference from the sample matrix. However, LPME methods can be time-consuming, and equilibrium may not be achieved even after prolonged extraction times in many cases (7-8).

Shahdousti *et al*. (2007) introduced a method for extracting VPA from human blood serum samples using HS-LPME with GC-FID analysis. This technique eliminates the need for solvent evaporation or VPA derivatization before injection. In their study, four organic solvents (1-decanol, benzyl alcohol, 1-octanol, and n-dodecane) were investigated as potential extractor solvents. The procedure involves suspending a 2 µL droplet of n-dodecane (selected organic solvent) in a 1 mL serum sample. Results demonstrated the effectiveness of HS-LPME in extracting VPA from human serum samples, achieving a LOD of 0.8 $\mu g.mL^{-1}$. Furthermore, the method was successfully applied to the analysis of valproate in pharmaceutical preparations (8).

DLLME

Dispersive liquid-liquid microextraction (DLLME) is an effective extraction and enrichment technique that allows for simultaneous derivatization, extraction, and

preconcentration in a single step. DLLME utilizes a ternary component solvent system and offers several advantages, including simplicity, low cost, rapid operation, and eco-friendliness. In DLLME, a mixture of extraction solvent (low soluble in aqueous phase) and disperser solvent (soluble in both extraction solvent and aqueous phase) is injected into the aqueous solution of analytes, forming microdroplets of extracting solvent, which increases the contact surface. After centrifugation, the high-density droplets of extraction solvent settle down, and this sedimented phase is further subjected to analysis. The use of a disperser solvent in DLLME reduces the sample polarity, leading to increased analyte solubility in the sample solution and a decrease in extraction performance, especially in low volumes of biological samples (7, 9, 11).

Fazeli-Bakhtiyari *et al*. (2015) established a DLLME method coupled with GC-FID analysis for the determination of VPA in human plasma. To optimize the extraction process, different solvents were tested. Dichloromethane, tetrachloroethylene, chloroform, and carbon tetrachloride were considered extraction solvents due to their higher density than water, facilitating easy removal from the bottom of the vial after centrifugation. Additionally, disperser solvents such as methanol, acetonitrile, acetone, and tetrahydrofuran were evaluated. The method involves adding a mixture of extraction solvent (40 µL chloroform) and disperser (1 mL acetone) to 10 mL of diluted plasma containing VPA. The LOD was determined to be 3.2 $\mu g.mL^{-1}$, and the LLOQ was 6 $\mu g.mL^{-1}$. To access the method applicability, a real blood sample was obtained from a male patient who had ingested VPA (125 mg) along with flurazepam, bipyridine, risperidone, and propranolol. The results indicated the method is efficient, with no interfering peaks observed at the VPA retention time (7).

Jain *et al*. (2015) developed a DLLME method for analyzing VPA in urine, utilizing a combination of methyl chloroformate (MCF) and DLLME procedure for simultaneous derivatization and extraction, followed by gas chromatography–tandem mass spectrometry (GC-MS/MS) analysis. The procedure involves the rapid injection of a mixture containing 200 µL of methanol (disperser solvent), 100 µL of TCE (extraction solvent), and 25 µl of MCF (derivatizing agent) into the aqueous sample using a syringe, resulting in a cloudy solution. This single-step method achieves derivatization, extraction, and preconcentration at room temperature, reducing the consumption of extraction solvent compared to traditional LLE and SPE methods. Additionally, using MCF for derivatization overcomes the drawbacks of silylation, such as prolonged reaction times and the need for moisture-free conditions. To determine the optimal analysis conditions, the efficacy of alkyl chloroformate reagents (ECF, MCF, and IBCF) in VPA derivatization was evaluated. The search for a suitable extraction solvent included testing solvents with higher density than water ($CHCl_3$, TCE, TeCE, and CB). Additionally, four commonly used disperser solvents (EtOH, MeOH, ACN, and acetone) underwent testing for their dispersing capabilities. The proposed method is characterized by its rapidity, with a total analysis time of only 15 minutes, encompassing derivatization, extraction, and GC-MS/MS analysis. It has proven to be sensitive, economical, and eco-friendly, achieving a LOD of 0.4 $ng.mL^{-1}$ and a LOQ of 1.4 $ng.mL^{-1}$ (9).

AALLME

A new method called air-assisted liquid-liquid microextraction (AALLME) was developed to enhance the performance of DLLME without the need for a disperser solvent. A small volume of the extraction solvent is transferred into the aqueous sample, and the mixture is repeatedly drawn into a glass syringe and reinjected, increasing the turbidity of the solution. AALLME achieves dispersion of the extraction solvent into the sample solution efficiently, improving analyte extraction with minimal solvent consumption (11, 20).

Feriduni *et al*. (2019) introduced a novel and efficient microextraction technique coupled with GC-FID for the isolation and quantification of 3-heptanone and VPA in plasma samples. The method necessitates an extractor solvent with higher density than water, excellent extraction performance for the chosen analytes, immiscibility with the aqueous phase, and favorable chromatographic behavior. To meet these criteria, 1,2–DCE, 1,1,2,2–TCE, chloroform, and carbon tetrachloride were assessed. Chloroform emerged as the optimal choice, exhibiting superior performance for the selected analytes. Subsequently, only 20 µL of chloroform was required for further experiments, thereby reducing the organic solvent quantity and enhancing environmental friendliness. The LOQ for 3-heptanone and VPA were determined to be 0.04 $\mu g.mL^{-1}$ and 0.2 $\mu g.mL^{-1}$, respectively. The LOD was established at 0.010 $\mu g.mL^{-1}$ for 3-heptanone and 0.057 $\mu g.mL^{-1}$ for VPA. The developed procedure was successfully applied to analyze plasma samples obtained from 70 children with epilepsy undergoing valproate treatment (11).

SPME

Solid phase microextraction (SPME) is based on the exposure of a fused silica fiber coated with a polymer to the dispersed volatile and semi-volatile compounds from a sample, developed in the early 1990s by Belardi and Pawliszyn, which includes simultaneous extraction and preconcentration of analytes from aqueous samples or the headspace (HS-SPME) of the samples. The desired substances adhere to the fiber through adsorption, and subsequently, desorption occurs using solvent or heat. In HS-SPME, the fiber is introduced into a sealed flask containing the sample, and heating is applied to release volatile compounds into the headspace above the sample. It offers advantages like simplicity, speed, easily automated and compatibility with GC or high-performance liquid chromatography (HPLC). The ability of SPME in hyphenation with various analytical methods is one of the most important of its advantages (14, 21).

The efficiency of SPME is determined by the distribution of analytes between the sample matrix and the fiber

coating. The fiber coating is a crucial component of the SPME device. Over time, various novel coatings have been developed for extracting different types of compounds, supplementing the commercially available SPME fibers. However, these fibers have certain limitations, including fragility, sensitivity to complex matrices, high cost, sample carryover problems, limited lifespan, degradation with use, bleeding of the SPME coating into the GC injector, and a limited selection of stationary phases for the fibers. Despite its drawbacks, SPME remains a useful technique for sample extraction in many applications (7-8, 11, 16).

Deng *et al.* (2006) demonstrated a method of quantification of VPA in human plasma, collected from three subjects after the administration of a single 500 mg oral dose of divalproex sodium, based on a rapid (less than 2 min) water-phase derivatization followed by HS-SPME and GC-MS. The VPA ethyl ester formed was headspace-extracted and simultaneously concentrated using the SPME technique where five different fibers were tested (PDMS, DVB/CW, PDMS/DVB, PA, and CAR/PDMS). PDMS fiber showed the maximum peak area of VPA ethyl ester, with an LOQ of 0.3 $\mu g.mL^{-1}$ (3).

Farajzadeh *et al.* (2009) developed a technique to extract VPA from human serum and pharmaceutical formulations using a homemade SPME fiber consisting of a hollow-fiber coated wire. Samples were prepared without derivatization and headspace extracted, then analyzed using capillary gas chromatography. The technique was successfully applied to samples from epileptic patients, with a LOD for VPA found to be 0.085 $\mu g.mL^{-1}$ in solution and 1.7 $\mu g.mL^{-1}$ in serum (14).

Matin *et al*. (2013) applied a nano-structured SPME fiber, composed of a nanosheet thin film based on Zn/Al LDH-TiO_2 composite, for VPA extraction from human serum and pharmaceutical formulations. The extraction process involved exposing the fiber to the samples headspace. The method was applied to spiked human serum samples, tablets and syrup samples, then the LOD was found to be 0.07 $\mu g.mL^{-1}$. Compared with previous articles, the LOD of the method using the proposed fiber was improved (22).

BioSPME

Conventional SPME automation systems lack the capability for parallel processing of multiple samples and often demand extended desorbing times. In contrast, the newly introduced SPME LC Tips, also known as BioSPME, offer an innovative approach. These tips consist of inert support coated with reversed-phase silica. The potential benefits of SPME LC Tips for drug extraction from biofluids are numerous, including operational simplicity, easy automation, reduced organic solvent consumption, and the ability to process multiple samples simultaneously, making them an attractive option (10).

Schaefer *et al.* (2021) developed and validated an assay for measuring VPA in serum using BioSPME technique, with subsequent GC/MS analysis. In this method, BioSPME LC Tips C18 are directly immersed into acidified serum, followed by agitation and desorption in methanol. The resulting methanolic extracts are then directly injected into the GC/MS, with calibrator samples ranging from 10 to 150 $\mu g.mL^{-1}$. To evaluate the effectiveness of the assay, 41 serum specimens submitted for routine VPA testing were processed using both LLE and BioSPME extraction under the same GC–MS conditions. A comparison of the methods revealed that the BioSPME LC Tips C18 sample preparation yielded clinically equivalent concentrations with a smaller amount of solvent. This highlights the efficiency of the BioSPME technique in the extraction process for VPA analysis in serum (10).

QuEChERS

Starting from the mid-2000s, the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) method was developed for pesticide extraction in food samples. This method is known for its simplicity, low cost, high efficiency, and limited number of steps. As a result, modified QuEChERS procedures have been successfully employed for the extraction of polar, moderately polar, and non-polar compounds in biological samples (23).

Mizuno and collaborators (2018) developed a method for the analysis of valproate in human plasma samples by QuEChERS extraction and gas chromatography–tandem mass spectrometry (GC-MS/MS). The entire QuEChERS extraction process, which included sample dilution (0.2 ml of human plasma with 1.3 ml of distilled water), QuEChERS extraction (400 mg of magnesium sulfate and 100 mg of sodium acetate), dSPE cleanup (containing 150 mg of magnesium sulfate and 50 mg of octadecylsilyl silica gel, C18), evaporation, and reconstitution (40 µl of ethyl acetate), was completed in approximately 30 minutes, using a total solvent volume of 1.0 mL of acetonitrile. For validation, drug-free whole blood samples were sourced from healthy volunteers, and the method demonstrated an LOD of 10 $ng.mL^{-1}$ and an LLOQ of 50 $ng.mL^{-1}$. The efficacy of the developed method was validated by applying it to the measurement of valproate in human plasma samples post-oral administration and in other materials (blood, urine, brain) in a postmortem case. This validation demonstrated the practical applicability of the method to real human plasma samples (23).

SALLE

Salting-out liquid-liquid extraction (SALLE) is a sample preparation technique used to concentrate and purify analytes from complex matrices such as blood, urine, or biological fluids before instrumental analysis. This method is a variation of liquid-liquid extraction (LLE), where the addition of an inorganic salt to the sample induces the separation of the aqueous and organic phases, facilitating the extraction of analytes into the organic phase. This technique has been effective in extracting various compounds from biomatrices, including polar ones. SALLE offers advantages of speed, environmental friendliness, and cost-efficiency compared to traditional liquid-liquid extraction and SPE methods (25, 26).

In 2021, Lipska *et al*. developed a highly sensitive GC/MS method for VPA quantification, employing pre-column esterification with methanol followed by hexane extraction. The derivatization process consisted of adding 2 mL of 10% sulfuric acid in methanol to each sample, followed by heating. Subsequently, 1 mL of saturated sodium bicarbonate solution and 1 mL of hexane were introduced to each sample for extraction. The method achieved a lower limit of quantification (LLOQ) of 0.075 $\mu g.mL^{-1}$ and a limit of detection (LOD) of 0.026 $\mu g.mL^{-1}$. The calibration curves demonstrated linearity within the concentration range of 0.075–15.0 $\mu g.mL^{-1}$. The accuracy presented excellent results, ranging from 96.07 to 106.61% (4). Although the authors obtained extremely low limits, the high sensitivity may represent a limitation in the case of VPA. In this case, the linearity obtained was up to 15 $\mu g.mL^{-1}$, while the therapeutic range goes from 50 to 100 $\mu g.mL^{-1}$. Therefore, real samples would need to be diluted for possible quantification.

Extraction methods for DBS

Testing therapeutic drug levels in dried blood spots (DBS) on paper collected by finger prick is a novel and emerging option for drug monitoring. DBS is an economical, simple, patient-friendly and easy-to-use method for blood collection, enabling patients to easily collect samples at home or in outpatient settings requiring only a small capillary blood volume from a finger prick, making it minimally invasive (2). Compared to conventional methods, utilizing DBS cards for therapeutic drug monitoring offers several advantages: patients can easily collect their own samples with a collection kit; monitoring can be done at any time and place; there are no biohazards involved in sample transfer; good stability of many analytes (12, 24). Despite the numerous advantages of the DBS sampling method, one of the challenges is the need for studies that evaluate the correlation of substance concentrations in venous and capillary blood, and it also presents significant challenges in detecting targets within the samples. Due to the low content of whole blood stored in DBS, the detection technique must be highly sensitive and capable of overcoming the matrix effects of blood. Additionally, the compounds in DBS need a complex elution or extraction process, and the efficiency of desorption directly impacts the analytical sensitivity (2). Instrumental chromatographic methods, matrix used and extraction methods are summarized in Table 2.

Table 2. Summary of instrumental chromatographic analysis described for VPA collected using DBS.

Extraction	Method	Chromatographic conditions	Matrix	Derivatization	Linear range ($\mu g.mL^{-1}$)	Ref.
Headspace	GC-MS	HP-5MS column (60 m × 0.25 mm i.d. × 0.25 µm), initial temperature of 70◦C, inlet temperature 260◦C, helium at 1 mL/min and the split ratio was 10:1	blood	-	1-200	2
LLE	GC-MS	CP-WAX column (30m× 0.25 mm i.d. × 0.25 µm), helium at 1 ml/min, column temperature was held at 80ºC for 2 min and then increased at 40ºC/min until 250ºC	blood	-	5-250	12
UALE[1]	GC-MS	DB-5 ms capillary column (30 m × 0.25 mm i.d., 0.25 µm), split mode, helium at 1,5 mL/min, column temperature 50 °C increased to 300 °C at a rate of 20 °C/min	plasma	MSTFA	10-200	24

[1]ultrasound-assisted liquid extraction

In 2019, Guo *et al*. introduced an innovative approach utilizing ink auxiliary headspace GC-MS for detecting sodium valproate in DBS from epilepsy patients. This method eliminates the need for additional solvent extraction or elution steps. The study began by comparing different processing methods for DBS, evaluating four types of black substances: fountain pen carbon black ink, prepared Chinese ink, black pencils with varying blackness, and ink for printing. Furthermore, various parameters of headspace sampling were optimized, including the size of filter paper, types of infiltrating solvent, volume, and additive, as well as the temperature of headspace sampling. Acetone was identified as the optimal solvent when compared to methanol, ethanol, and acetonitrile, which required only 50 µL volume. Additionally, 0.4% formic acid was chosen as an additive to enhance the desorption of sodium valproate through its ion inhibition effect. The incorporation of carbon black ink in the method enhances heat absorption and dissociation, resulting in improved headspace sampling quality. The application of headspace GC-MS offers several advantages for detecting DBS, such as the separation of volatile components from complex matrices and the simple desorption of samples through heat. DBS samples collected from 29 epilepsy patients undergoing sodium valproate treatment at Xuzhou Central Hospital were analyzed using this technique. The developed method enables the quantitative analysis of sodium valproate in DBS obtained from finger prick samples, providing ease in storage, and transportation, facilitating high-throughput clinical testing. The LOQ for sodium valproate in this method was established at 200 $ng.mL^{-1}$ (2).

Rhoden and colleagues (2014) developed a methodology for quantifying VPA in 6 mm punches of Whatman 903 paper, equivalent to approximately 12 µL of blood, utilizing GC-MS. The procedure involved transferring

these paper discs to microtubes, followed by the addition of an extraction solution comprising acetonitrile and methanol (1:3, v/v) with an internal standard (cyclohexanecarboxylic acid). This method, applicable to TDM, particularly proves beneficial in resource-constrained settings such as developing countries, with a LOQ of 5 $\mu g.mL^{-1}$. The DBS assay was applied to 17 samples collected from a neurology service in Brazil. These samples were obtained approximately 30 minutes before the scheduled administration of the next VPA doses. The results showed that VPA concentrations in serum and in DBS were highly correlated, when compared with venous blood samples collected within 10 minutes after the DBS finger prick, with a mean ratio of 1.883 and r= 0.9948 (12).

In 2014, a study conducted by Ikeda *et al.* applied the use of dried spots for quantify VPA and gabapentin in plasma using GC-MS. The study focused on utilizing dried plasma spots obtained from patients receiving in-home medical care. Two types of cards, Whatman FTA DMPK-A and Bond Elut DMS, were compared in terms of spot size, extraction efficiency, and drug stability. The extraction process involved using methanol, and the resulting residue underwent trimethylsilylation with MSTFA after evaporation. Both cards demonstrated a LOQ of 10 $\mu g.mL^{-1}$ for VPA. The proposed method holds significance for therapeutic drug monitoring in in-home medical patients. The emphasis lies on the simplicity, ease, and dry sampling method, making it particularly relevant for this context (24).

Conclusions

In this review, 19 studies that used gas chromatography for the determination of VPA in biological samples were described. Of these studies, the most used extraction methods are LLE, LPME, and SPME and related extractions. While there are a similar number of articles employing LLE and LPME, the ecological perspective favors LPME over LLE. This preference appears from the substantial solvent volumes used in LLE, making it less environmentally friendly. As for SPME, new protocols, including the use of new extractor phases, are described. Other approaches such as dried spots, a new method of collecting material prior to extraction, are emerging alternatives, both in the analytical scenario and in terms of ease of collection in the TDM scenario. The choice of extraction protocol is always conditioned by the objectives of the proposed method, the type of sample, and the analytical capacity of the laboratory.

Acknowledgments/Sources of funding

This study was supported by Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS/MS/CNPq 08/2020–PPSUS, grant process 21/2551-0000101) and by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil, Finance Code 001).

Conflict of interest

The authors declare no conflicts of interest.

References

1. Meyer MR, Welter J, Weber AA, Maurer HH. Development, Validation, and Application of a Fast and Simple GC–MS Method for Determination of Some Therapeutic Drugs Relevant in Emergency Toxicology. Ther Drug Monit. 2011; 33:649–653.
2. Guo MZ, Shao L, Chen X, Li HJ, Wang L, Pan YJ, et al. Assay of dried blood spot from finger prick for sodium valproate via ink auxiliary headspace gas chromatography mass spectrometry. J Chromatogr A. 2019 Sep 13; 1601:335–339.
3. Deng C, Li N, Ji J, Yang B, Duan G, Zhang X. Development of water-phase derivatization followed by solid-phase microextraction and gas chromatography/mass spectrometry for fast determination of valproic acid in human plasma. Rapid Commun Mass Spectrom. 2006; 20(8):1281–1287.
4. Lipska K, Gumieniczek A, Pietraś R, Filip AA. HPLC-UV and GC-MS Methods for Determination of Chlorambucil and Valproic Acid in Plasma for Further Exploring a New Combined Therapy of Chronic Lymphocytic Leukemia. Molecules. 2021 May 13; 26(10):2903.
5. Leis HJ, Windischhofer W, Rechberger GN, Fauler G. Synthesis of [18O2]valproic acid and its use as an internal standard for the quantitative measurement by gas chromatography–electron ionization mass spectrometry. Journal of Chromatography B. 2003; 784:69–75.
6. Chateauvieux S, Morceau F, Dicato M, Diederich M. Molecular and therapeutic potential and toxicity of valproic acid. Journal of Biomedicine and Biotechnology. 2010 Jul 29; 2010.
7. Fazeli-Bakhtiyari R, Panahi-Azar V, Sorouraddin MH, Jouyban A. Determination of valproic acid in human plasma using dispersive liquid-liquid microextraction followed by gas chromatography-flame ionization detection. Iranian Journal of Basic Medical Sciences. 2015; 18(10):979-988.
8. Shahdousti P, Mohammadi A, Alizadeh N. Determination of valproic acid in human serum and pharmaceutical preparations by headspace liquid-phase microextraction gas chromatography-flame ionization detection without prior derivatization. Journal of Chromatography B. 2007 May 1; 850(1-2):128–133.
9. Jain R, Gupta MK, Chauhan A, Pandey V, Reddy Mudiam MK. Ultrasound-assisted dispersive liquid-liquid microextraction followed by GC-MS/MS analysis for the determination of valproic acid in urine samples. Bioanalysis. 2015 Oct; 7(19):2451–2459.
10. Schaefer VD, de Lima Feltraco Lizot L, Hahn RZ, Schneider A, Antunes MV, Linden R. Simple determination of valproic acid serum concentrations

using BioSPME followed by gas chromatography-mass spectrometric analysis. Journal of Chromatography B. 2021 Mar 15; 1167:122574.
11. Feriduni B, Barzegar M, Sadeghvand S, Shiva S, Khoubnasabjafari M, Jouyban A. Determination of valproic acid and 3-heptanone in plasma using air-assisted liquid-liquid microextraction with the assistance of vortex: Application in the real samples. Bioimpacts. 2019 Mar 8; 9(2):105–113.
12. Rhoden L, Antunes MV, Hidalgo P, Álvares da Silva C, Linden R. Simple procedure for determination of valproic acid in dried blood spots by gas chromatography-mass spectrometry. J Pharm Biomed Anal. 2014 Aug 5; 96:207–212.
13. Gu X, Yu S, Peng Q, Ma M, Hu Y, Zhou B. Determination of unbound valproic acid in plasma using centrifugal ultrafiltration and gas chromatography:Application in TDM. Anal Biochem. 2020 Jan 1; 588:113475.
14. Farajzadeh MA, Farhadi K, Matin AA, Hashemi P, Jouyban A. Headspace solid-phase microextraction-gas chromatography method for the determination of valproic acid in human serum, and formulations using hollow-fiber coated wire. Anal Sci. 2009 Jul; 25(7):875–879.
15. Khatibi, S. A., Hamidi, S., & Siahi-Shadbad, M. R. (2022). Application of liquid-liquid extraction for the determination of antibiotics in the foodstuff: recent trends and developments. Critical reviews in analytical chemistry, 52(2), 327-342.
16. Koller MF, Schmid M, Iten PX, Vonlanthen B, Bär W. Fatal intoxication with naftidrofuryl. Leg Med. 2009 Sep; 11(5):229–233.
17. Namera, A., Uekusa, K., Saito, T., Yoshimoto, K., Ishiuchi, N., Murata, K., & Nagao, M. (2022). A method for determining valproic acid in human whole blood and urine via gas chromatography-mass spectrometry and small-scale inter-laboratory trial. Legal Medicine, 59, 102133.
18. Aly, A. A., & Górecki, T. (2020). Green approaches to sample preparation based on extraction techniques. Molecules, 25(7), 1719.
19. Stephenson JB, Flater ML, Bain LT. Analysis of Valproic Acid, Salicylic Acid and Ibuprofen in Whole Blood by GC-MS. J Anal Toxicol. 2016 Oct; 40(8):649–652.
20. Farajzadeh MA, Mogaddam MRA. Air-assisted liquid-liquid microextraction method as a novel microextraction technique; application in extraction and preconcentration of phthalate esters in aqueous sample followed by gas chromatography-flame ionization detection. Anal Chim Acta. 2012 May 30; 728:31–38.
21. Birk L, dos Santos BP, Schwarz P de S, Eller S, de Oliveira TF. Green chemistry: Developments and perspectives for sample treatment in forensic methodologies. WIREs Forensic Science. 2023 Nov; 5(6).
22. Matin AA, Biparva P, Amanzadeh H, Farhadi K. Zinc/Aluminum layered double hydroxide-titanium dioxide composite nanosheet film as novel solid phase microextraction fiber for the gas chromatographic determination of valproic acid. Talanta. 2013 Jan 15; 103:207–13.
23. Mizuno S, Lee XP, Fujishiro M, Matsuyama T, Yamada M, Sakamoto Y, et al. High-throughput determination of valproate in human samples by modified QuEChERS extraction and GC-MS/MS. Leg Med. 2018 Mar; 31:66–73.
24. Ikeda, K., Ikawa, K., Yokoshige, S., Yoshikawa, S., & Morikawa, N. (2014). Gas chromatography–electron ionization–mass spectrometry quantitation of valproic acid and gabapentin, using dried plasma spots, for therapeutic drug monitoring in in-home medical care. Biomedical Chromatography, 28(12), 1756-1762.
25. Lee, M. K., & Jung, M. Y. (2023). A highly efficient strategy for simultaneous analysis of eleven monomeric hydroxylated-stilbenes in grape wines and juices by a salting-out assisted liquid–liquid (SALLE) extraction combined with a high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). Food Chemistry, 426, 136661.
26. Tang, Y. Q., & Weng, N. (2013). Salting-out assisted liquid–liquid extraction for bioanalysis. Bioanalysis, 5(12), 1583-1598.
27. Shnayder, N. A., Grechkina, V. V., Khasanova, A. K., Bochanova, E. N., Dontceva, E. A., Petrova, M. M., ... & Nasyrova, R. F. (2023). Therapeutic and toxic effects of valproic acid metabolites. Metabolites, 13(1), 134.