Journal of Reports in Pharmaceutical Sciences 2018, 7(1), 27-43

Gas Chromatographic Analysis of Sodium Valproate in Plasma and Urine after Air Assisted Liquid-Liquid Microextraction

Maryam Abbaspour[a], Mir Ali Farajzadeh[b], Maryam Khoubnasabjafari[c], Sajad Haririan[d], Abolghasem Jouyban[a,e]*

[a]Pharmaceutical Analysis Research Center and Faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran
[b]Department of Analytical Chemistry, Faculty of Chemistry, University of Tabriz, Tabriz, Iran
[c]Lung and Tuberculosis Diseases Research Center, Tabriz University of Medical Science, Tabriz, Iran
[d]Neurology Department, Aalinasab Hospital, Tabriz, Iran
[e]Kimia Idea Pardaz Azarbayjan (KIPA) Science Based Company, Tabriz University of Medical Sciences, Tabriz, Iran

ARTICLE INFO

Article Type:
Research Article

Article History:
Received: 2017-08-05
Revised: 2017-09-25
Accepted: 2017-10-01
ePublished: 2017-10-15

***Keywords*:**
Air-assisted dispersive liquid–liquid microextraction
Biological sample
Gas chromatography
Plasma
Sodium valproate
Urine

A B S T R A C T

Rapid, highly efficient, and reliable liquid–liquid microextraction (LLME) methods followed by gas chromatography-flame ionization detection were developed for the extraction, preconcentration, and determination of valproate in human plasma and urine samples. Proteins of plasma sample are precipitated by adding methanol and urine sample was diluted prior to performing the microextraction procedures. Fine organic solvent droplets were formed by repeated suction and injection of the mixture of sample solution and extraction solvent into a test tube with a glass syringe. After extraction, phase separation was performed by centrifuging and the enriched analytes in the sedimented organic phase were determined by the separation system. The main factors influencing the extraction efficiency including extraction solvent type and volume, salt addition, pH, and extraction times are investigated. Under the optimized conditions, the proposed method showed good precision (relative standard deviation less than 8%). Limits of detection and lower limits of quantification for valproate were obtained in the ranges of 0.05–0.22 and 0.1–0.5μg mL^{-1}, respectively. The linear ranges were 0.5-500 and 0.1-200 μg mL^{-1}in plasma and urine, respectively ($r^2 \geq 0.9995$). The relative recoveries varied from 98-102 % and 93-100 %, respectively for plasma and urine samples. The mean relative standard deviations for intra-assay and inter-assay precisions were 3.4 % and 6.0 %, respectively. Preconcentration factors were in the range of 7-44. Good recoveries (55–86%) were obtained for the spiked samples. The proposed method was successfully used to analyze plasma and urine samples of epileptic receiving sodium valproate.

***Corresponding Author**: **Abolghasem Jouyban, E-mail:** ajouyban@hotmail.com

Introduction

Analysis of antiepileptic drugs in biological fluids is an important issue in modern therapeutics and monitoring of their concentrations are commonly done to adjust the drug dosage and follow up of treatment process. Plasma and urine are the most frequently used biological samples and simple, sensitive and low cost methods are highly in demand in clinical chemistry laboratories. Different analytical methods used for analysis of these drugs have been reviewed [1, 2]. These methods vary from simple and low-cost spectroscopic methods [3] to a complicated and expensive liquid/gas chromatographic mass spectroscopic methods [4].

Valproic acid (2-propylpentanoic acid, $C_8H_{16}O_2$) is a C_8-branched carboxylic acid and a colorless liquid that is slightly miscible with water and very soluble in organic solvents and possesses a pK_a of 4.6 [5]. Sodium valproate is a well-established anticonvulsant drug that has been increasingly used in the treatment of many forms of generalized epilepsy [6]. Epilepsy is the most common serious neurological disorder affecting 1% of the world population [7]. Since valproate is highly bound to albumin (approximately 80–95%), only a small fraction exists in free form [8].

The therapeutic concentration of VPA ranges from 50 to 100 μg mL^{-1} [9, 10]. Valproate is rapidly absorbed, attaining maximal blood levels within four hours after oral administration. It is quickly distributed to tissues and liver (95% metabolized) [11]. Valproate has complex metabolic pathways despite its simple structure [12]. It is almost completely eliminated by metabolism, with less than 4% of an administered dose excreted unchanged into the urine [13]. Monitoring of VPA levels in patient plasma and body fluids on a routine basis is essential when there are changes in VPA dose, concomitant medication or clinical condition of patient [14].

There are several analytical methods reported in the literature for the quantification of VPA in biological matrices either alone or in combination with other drugs. These include capillary electrophoresis (CE) [15, 16], high-performance liquid chromatography (HPLC) with MS detection [9, 17-20], UV detection [2, 21-24] and fluorescence detection [25], and gas chromatography (GC) [26-34]. Some sample preparation techniques based on liquid–liquid extraction (LLE) [2, 21], solid phase extraction (SPE) [9], solid-phase microextraction (SPME) [35], liquid-phase microextraction (LPME) [31], and dispersive liquid–liquid microextraction (DLLME) [16, 3-39] have been developed. LLE and SPE require a large amount of organic solvents and are time-consuming. SPME is a solvent-free process which despite of its numerous advantages, it also suffers from some drawbacks: its fiber is fragile and has a limited lifetime and the sample carryover is also a problem [40]. LPME is a solvent-minimized sample preparation approach in which only several microliters of an extracting solvent is required [41]. In 2006, a novel liquid phase microextraction method i.e. DLLME was introduced by Assadi and co-workers [42]. This high-performance microextraction method employs a ternary component solvent system, in which extraction and disperser solvents are rapidly injected into the aqueous sample to form a cloudy solution. The analytes enriched in the dispersed fine droplets of the extraction solvent is then separated by centrifuging. DLLME has been proved to be a simple, low-cost, and fast method using the small amount of the extraction solvent and the low sample volume together with a high preconcentration factor (PF) for the analysis of different analytes. The use of relatively larger volumes of a disperser solvent is the most significant drawback of DLLME, because it reduces the polarity of aqueous phase which leads to increase the solubility of analytes into aqueous phase and decreases extraction efficiency.

In order to resolve above mentioned problem, some disperser solvent-free techniques such as ultrasound- assisted emulsification microextraction (USAEME) [43] and vortex-assisted liquid–liquid microextraction (VALLME) [44] were developed, in which the extraction solvent is dispersed into an aqueous sample through ultrasound irradiation or vortexing. However, without the use of an organic disperser solvent, the process of forming cloudy solution typically takes a significantly longer time than conventional DLLME method. In 2012, air-assisted liquid–liquid microextraction (AALLME) [45] was developed, which is a new version of the DLLME method. In

AALLME, a few microliters of an extraction solvent are transferred into an aqueous phase containing the analytes. Fine organic solvent droplets are formed by repeated suction and injection of the mixture of the aqueous sample solution and the extraction solvent into a test tube with a glass syringe. By performing the predetermined cycles the turbidity of solution is increased and analytes are extracted into the organic phase. After centrifugation of cloudy solution, the extractant is settled down in the bottom of the centrifuge tube and used for further analysis. AALLME is a disperser solvent-free technique which is completely rapid.

In the present study, simple and rapid microextraction methods with improved sensitivity and reproducibility for the determination of valproate in human plasma (using DLLME) and in urine (using AALLME) followed by GC-flame ionization detection (FID) were proposed. The effect of some experimental parameters, including the type and volume of the extraction solvent, salt addition, pH, and extraction times on the extraction efficiency are studied and optimized. The performance of the optimized method was then evaluated and successfully applied to determine valproate in biological samples.

Methods and Materials

Chemicals

Sodium valproate was kindly provided by RouzDarou Pharmaceutical Co. (Tehran, Iran). The tested extraction solvents were supplied from the following sources: carbon tetrachloride, 1,2–dichloroethane (1,2–DCE), 1,1,1–trichloroethane (1,1,1–TCE), and 1,1,2-trichloroethane (1,1,2–TCE) were from Merck (Darmstadt, Germany), 1-bromo-2-chloroethane was from Janssen Chimica (Beerse, Belgium), and chloroform ($CHCl_3$) was obtained from Scharlau (Barcelona, Spain). HPLC–grade methanol, sodium chloride, hydrochloric acid, and sodium hydroxide were purchased from Merck. De–ionized water (Shahid Ghazi Pharmaceutical Company, Tabriz, Iran) was used for preparation of aqueous solutions. A stock solution of sodium valproate (100 mg L^{-1}) was prepared in methanol and stored in a refrigerator at 4 °C. Working standard solutions were daily prepared by appropriate dilutions of the stock solution with de–ionized water. A standard solution of sodium valproate (100 mg L^{-1}) in chloroform was injected into GC-FID (three times in a day) and the obtained analytical signals (peak areas) were used for the calculation of PFs and extraction recoveries (ERs). A phosphate buffer (1.0 mol L^{-1}) was prepared by dissolving 39.0 g of sodium dihydrogen phosphate dehydrate ($NaH_2PO_4 \cdot 2H_2O$) (Merck) in 1000 mL deionized water, and its pH was adjusted at 2.0 by adding HCl 1.0 mol L^{-1}.

Samples

Plasma samples

Drug-free human plasma samples were obtained from the Iranian Blood Transfusion Research Center (Tabriz, Iran) and frozen in polypropylene microtubes (2-mL fractions) at -20 °C until analysis. Also blood samples were obtained from 16 patients receiving the drug who had signed consent forms approved by the ethics committee, Tabriz University of Medical Sciences. 5 mL blood was taken in the heparinized tube 12 h after the last administration of sodium valproate. Plasma samples were immediately separated by centrifugation at 5000 rpm for 10 min. To precipitate the proteins, 100 µL of plasma sample was mixed with 200 µL methanol. Then the obtained mixture was vortexed for 15 s and centrifuged for 7 min at 3000 rpm. Then 100 µL of the supernatant phase was removed and diluted with 4.9 mL sodium phosphate buffer (1 mol L^{-1}, pH 2.0) and used for further DLLME procedure.

Urine samples

Drug-free urine samples were collected from healthy volunteers. Also urine samples were obtained from 16 patients mentioned in previous section. Samples were collected in polypropylene tubes (2 mL fractions) and stored at -20 °C until analysis. The collected urine samples were centrifuged at 3000 rpm for 7 min. To reduce the matrix effect of urine sample the supernatant was

diluted 5-fold with phosphate buffer (1.0 mol L^{-1}, pH 2.0) and then were subjected to the microextraction procedure.

AALLME procedure

5 mL of diluted plasma or urine sample was placed into a 10-mL glass test tube with conical bottom. Chloroform (75 µL) as an extraction solvent was added to the tube and then the mixture was repeatedly aspirated into a 5–mL glass syringe and then was expelled into the tube. This caused the solution to become turbid. The procedure was repeated for 4 times. After this process, the mixture was centrifuged at 3000 rpm for 7 min and fine droplets of the extractant were settled down in the bottom of the centrifuge tube (10 ± 1 µL). Finally, 1 µL of the sedimented phase was removed and injected into GC system for analysis.

Instrumentation

GC analysis of VPA was carried out using an Agilent 7890A gas chromatograph (Agilent Technologies, CA, USA) equipped with a split/splitless inlet system operated at 300 ºC in a splitless mode (sampling time of 1 min) and an FID. Nitrogen (99.999%, Gulf Cryo, United Arab Emirates) was used as the carrier gas (at a constant flow of 1.2 mL min^{-1}) and make up gas (25 mL min^{-1}). Chromatographic separation was achieved on an HP–5 capillary column (30 m × 0.32 mm i.d. with a 0.25 µm stationary film thickness) (Hewlett-Packard, Santa Clara, USA). The oven temperature was programmed from 50 °C (held for 2 min) to 210 °C at a rate of 10 °C min^{-1} and held at 210 °C for 3 min; then, the temperature was raised with a rate of 15 °C min^{-1} to a final temperature of 290 °C that was held for 1 min. Chem Station software was used for data acquisition and processing. A 1-µL microsyringe (zero dead volume, Hamilton, Switzerland) was used for the injection of samples into GC. Injection volume was 1 µL. The FID temperature was maintained at 300 °C. Hydrogen gas was generated with a hydrogen generator (GLAIND-2200, Dani, Italy) for FID at a flow rate of 40 mL min^{-1}. Air flow rate for FID was 400 mL min^{-1}. A vortex from Labtron Company (Tehran, Iran) was used in sample preparation. A Metrohm pH meter model 744 (Herisau, Switzerland) was used for pH measurements. Sigma centrifuge (Osterode, Germany) was used in protein precipitation step and Hettich centrifuge (Tuttlingen, Germany) was used for accelerating phase separation.

Analytical parameters

Two main parameters, namely PF and ER, have been employed for evaluation of the proposed method. PF is defined as the ratio between the analyte concentration in the sedimented phase (C_{sed}) and the initial concentration of analyte (C_0) within the sample:

$$PF = \frac{C_{sed}}{C_0} \quad (1)$$

C_{sed} is obtained from a suitable calibration graph prepared or by comparison with peak areas of the analytes obtained by directly injection the standard solution prepared in the extraction solvent. ER is defined as the percentage of the total analyte amount (n_0) which is extracted into the sedimented phase (n_{sed}):

$$ER = \frac{n_{sed}}{n_0} \times 100 = \frac{C_{sed} \times V_{sed}}{C_0 \times V_{aq}} \times 100 = PF \times \frac{V_{sed}}{V_{aq}} \times 100 \quad (2)$$

Where V_{sed} and V_{aq} are volumes of the sedimented phase and aqueous solution, respectively.

Assay validation

For the validation of the recommended LLME methods in the determination of valproate under the experimental conditions, the related analytical characteristics were calculated by employing the peak areas. Method validation studies include all procedures which demonstrate that a method is suitable for its intended application. The validation process of the present method was carried out following the Food and Drug Administration (FDA) guidelines [46]. In order to do this, the calibration linearity, limit of detection (LOD), limit of quantification (LOQ), lower limit of quantification (LLOQ), upper limit of

quantification (ULOQ), intra- and inter-day precisions, accuracy, recovery, specificity and selectivity, stability (room temperature and freeze–thaw) and robustness of the proposed method were evaluated in plasma and urine samples. The mean of three calibration curves (produced on three different days) was used for linearity studies. All experiments were performed three times. LODs and LOQs were calculated on the basis of signal to noise ratio (S/N) of 3 and 10, respectively. S/N was estimated using measurements of the peak height relative to the baseline noise, and height values were consequently converted into concentrations through the height of the analyte peaks at the LLOQ. LLOQ and ULOQ terms are defined as the lowest and highest concentration level of calibration curve that can be measured with an acceptable accuracy and precision. The intra- and inter-day precision were evaluated by assaying the quality control (QC) samples at three concentration levels and expressed as relative standard deviations (RSD). The accuracy of method was also determined by calculating relative errors (RE %) using the following equation:

$$RE(\%) = 100 \times \left(\frac{\text{calculated conc.} - \text{nominal conc.}}{\text{nominal conc.}} \right) \quad (3)$$

ERs of the interested analytes were determined by comparing the peak area of the QC samples with that of the corresponding standard solution prepared in the extraction solvent. The relative recoveries were calculated as the ratio of the found concentration after extraction to the added concentration before extraction multiplied by 100. In the present study, specificity investigated by analyzing six different sources of blank plasma and urine samples under the optimal experimental conditions. Moreover, selectivity of the developed method was studied by analysis of samples spiked with some other co- administered antiepileptic drugs (AEDs) for potential interferences. The stability of analytes in samples was assessed by analyzing triplicate QC samples at different storage conditions: short term (12 h) room temperature and three freeze– thaw (-20 to 25°C) cycles. The concentrations following storage were compared with freshly prepared samples of the same concentrations. Furthermore, the robustness of the method was evaluated by partial varying of some effective parameters in AALLME method such as extraction solvent volume, sample solution ionic strength and its pH in three levels.

Results and discussion

In order to obtain the best extraction efficiency, some important experimental parameters that would influence the performance of LLME methods were investigated in details in the following sections. The parameters including extraction solvent type and volume, salt addition, pH, and extraction numbers were studied. To optimize the method, all extractions were initially carried out on human plasma spiked with sodium valproate then applied to spiked urine sample, and finally to samples taken from epileptic patients.

Selection of extraction solvent

The selection of extraction solvent is the most important experimental parameter of an LLME method. Generally, the extraction solvent has to possess insignificant solubility in water, high extraction capability of the interested analytes, and good chromatographic behavior. Also, it should have different density from water to enable phase separation after extraction. Based on these facts, some organic solvents named chloroform, 1-bromo-2-chloroethane, carbon tetrachloride, 1, 2-DCE, 1, 1,1–TCE, and 1, 1,2–TCE were examined. Different volumes of these solvents were tested to reach a volume of 10 ± 1 µL volume of the sedimented phase at the bottom of the test tube. The needed volumes for each solvent were chloroform 75 µL, 1-bromo-2-chloroethane 90 µL, carbon tetrachloride 35 µL, 1, 2-DCE 70 µL, 1,1,1–TCE 35 µL, and 1,1,2–TCE 45 µL. Comparison of the peak areas obtained with different extraction solvents (Figure 1) indicate that chloroform is the most effective extraction solvent among the tested solvents and gave the highest extraction efficiency for valproate. Therefore, it was selected as the suitable

extraction solvent for the subsequent experiments.

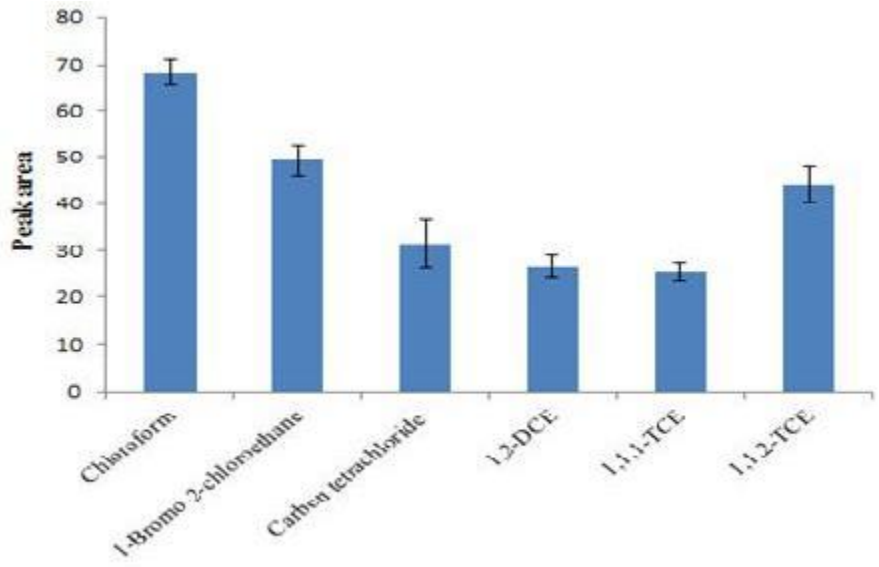

Fig. 1. Effect of extraction solvent kind.

Extraction conditions: phosphate buffer volume (C=1 mol L^{-1}, pH 2.0), 5 mL; sodium valproate concentration, 200 ng mL^{-1}; extraction solvent, chloroform (75 µL), 1-bromo-2-chloroethane (90 µL), carbon tetrachloride (35 µL), 1,2-DCE (70 µL), 1,1,1–TCE (35 µL), and 1,1,2–TCE (45 µL); extraction numbers, 5 times; centrifuge rate, 3000 rpm; and centrifuge time, 7 min. The error bars indicate the minimum and maximum of three determinations.

Extraction solvent volume

Extraction solvent volume is another important factor that can affect volume of the sedimented organic phase, extraction efficiency and repeatability of the results obtained. To examine effect of the extraction solvent volume on the extraction performance, experiments involving different volumes of chloroform (70, 75, 80, 85, 90, and 100 µL) were done with the same extraction procedure while the other experimental conditions were kept constant. The results reveal that by increasing the extraction solvent volume from 75 to 100 µL, the peak areas decrease due to increase in volume of the sedimented phase from 10 to 22 µL which in turn leads to decrease in analyte concentration into the organic phase and PFs, too. It is noted that in the case of 70 µL extraction solvent volume or less, removal of the sedimented phase was difficult and repeatability of the responses was also low. Therefore, further experiments were carried out with 75 µL of chloroform, which leads to obtain 10 ± 1 µL sedimented phase volume.

Effect of salt addition

Ionic strength affects the extraction efficiency by its influence on the solubility of analytes and viscosity of the aqueous phases. The effect of salt addition on the extraction efficiency of the LLME method was studied by adding sodium chloride in the range of 0–15 %, *w/v*, to the sample solution. Salt addition leads to an increase in volume of the sedimented phase by decreasing the solubility of extraction solvent into aqueous phase. Therefore, the experiments were performed using different volumes of the

extraction solvent to achieve 10 µL of the sedimented phase volume (75, 71, 67, 62, 57, and 50 µL for 0, 2.5, 5.0, 7.5, 10, and 15 % NaCl, *w/v*, respectively). By increasing NaCl concentration, analytical signals increase till 7.5 % and then remain almost constant. This effect can be attributed to decrease the solubility of analytes in the aqueous phase with increasing ionic strength due to salting out effect. Therefore the further experiments were performed in the presence of 7.5 %*w/v*, NaCl.

Effect of pH

The pH of the sample solution is an important parameter affecting the hydrolysis as well as solubility of the analytes in aqueous phase. The effect of sample pH was investigated within a pH range of 1.0–6.0 with adjusting pH using solutions of 0.1 M HCl. Valproic acid with a pK_a of 4.6 exists in neutral (un-ionized) form at low pH, and is completely ionized at pH higher than 6.6; thus, the pH value above 6.0 was not tested. Careful examination of the results reveal that at pH 1.0 the peak area decreased significantly compared to pH 2.0. The best extraction efficiency was obtained at pH 2.0; therefore, for subsequent experiments pH was adjusted to 2.0. To facilitate the pH adjustment, phosphate buffer (1.0 mol L^{-1}, pH 2.0) was used instead of HCl solution. The obtained results in both cases were similar.

Effect of extraction cycles number

In an AALLME method, formation of fine droplets of the extraction solvent dispersed into aqueous phase is performed by repeatedly sucking extraction solvent and sample solution mixture into a 5-mL glass syringe and then its injecting into a test tube. The numbers of suction/injection cycles are considered as the extraction cycles number. It was predictable that with increasing extraction cycles number, extraction efficiency would be increased and then remained constant. Therefore to obtain the equilibrium status, the extraction cycles number was studied in the range of 1–9 times. The results in **Figure 2** show that analytical signals increase with increasing the extraction cycles to 4 and then decrease. That is because in high extraction cycle numbers vaporization of the extraction solvent would be significant. Consequently, 4 times of extraction was selected for further studies. It is noted that this step is very rapid and takes less than 30 s.

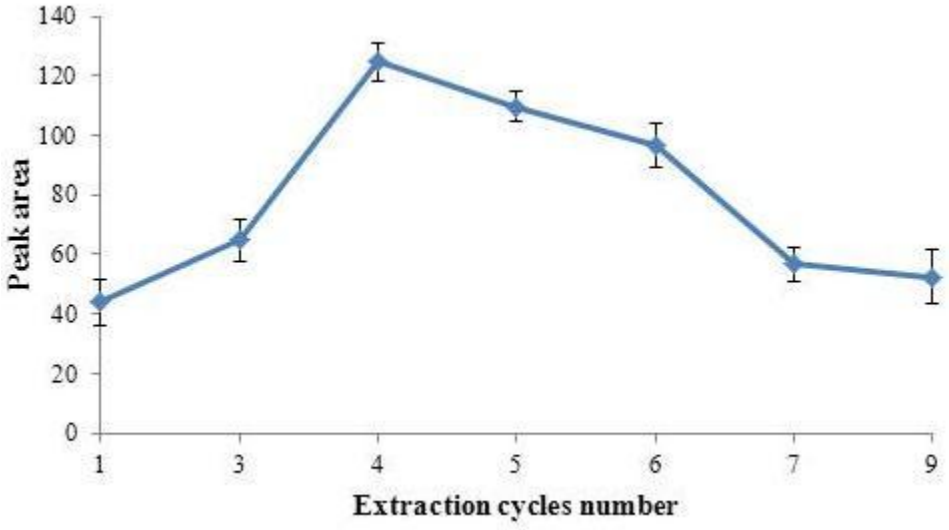

Fig. 2. Optimization of number of extraction cycles.

Extraction conditions: extraction solvent (chloroform) volume, 75 µL, pH 2.0, NaCl concentration 7.5 %, *w/v*.. The error bars indicate the minimum and maximum of three determinations.

Optimization of centrifugation time and speed

Centrifugation is substantial in order to obtain two separated phases after extraction. In order to achieve the best extraction efficiency, centrifugation time and speed were considered in the ranges of 3–9 min and 2000–6000 rpm, respectively. The obtained results showed that these parameters were less effective. Therefore, 3000 rpm and 7 min were selected as the optimal centrifuge rate and time, respectively, in the following studies.

Method validation

Linearity and calibration curves

After optimization of all parameters, the calibration curves were constructed in 3 different days at seven increasing levels and the average of three replicated curves was used for validation studies. The details of calibration curves and corresponding validation data (i.e., linear range, LOD, LOQ, LLOQ, and ULOQ) are presented in **Table 1**.

Table 1. Validation data of the proposed method for quantification of the sodium valproate in human plasma and urine sample.

Sample	LR[a]	Slope	Intercept	r^2 [b]	Number of data points	LOD[c]	LOQ[d]	LLOQ[e]	PF ± SD[f]	ER% ± SD[g]
Plasma	0.5-500	20.8	0.5	0.9999	7	0.22	0.73	0.5	0.3 ±7	3 ± 71
Urine	0.1-200	106.6	6.5	0.9996	7	0.05	0.16	0.1	3±44	4 ±66

[a] Linear range (μg mL^{-1}).
[b] Square of correlation coefficient.
[c] Limit of detection (S/N = 3) (μg mL^{-1}).
[d] Limit of quantification (S/N = 10).
[e] Lower limit of quantification (μg mL^{-1}).
[f] Preconcentration factor ± standard deviation (n = 3).
[g] Extraction recovery ± standard deviation (n = 3).

Assay precision and accuracy

Precision and accuracy of the method were assessed under the obtained conditions for both intra- and inter-days. These two parameters are expressed as the closeness of the individual measures of an analyte and deviation of mean test results from nominal concentrations, respectively. The repeatability and reproducibility of the proposed method, expressed as RSD %, were evaluated by performing the method on six repeated QC samples in a day (for intra–day assay) and four repeated QC samples in different days (for inter–days assay) at three concentration levels (low, medium, and high). All RSD % values were less than 8.0 %. The accuracy of the method was determined by calculating the percentage deviation observed in the analysis of QC samples and expressed as the relative error (RE %). Inter- and intra-assay precisions along with accuracy for QC samples were listed in **Table 2**. These results demonstrate that the values are within the acceptable range recommended by FDA, and, hence, the developed method is sufficiently accurate and precise.

Table 2. Precision and accuracy of the method for determination of the sodium valproate in human plasma and urine sample.

Sample	Nominal concentration (µg mL^{-1})	Intra-assay precision (RSD %[a]) (n = 6)	Accuracy (RE[b] %)	Inter-assay precision (RSD %) (n = 4)	Accuracy (RE %)
Plasma	0.5	6.2	-0.1	7.2	0.4
	50	2.4	2.5	2.6	3.1
	500	1.2	1.9	1.5	0.7
Urine	0.1	5.4	-2.5	7.0	-1.5
	10	4.3	-3.7	4.7	-5.2
	200	1.1	0.02	1.3	0.3

[a] Relative standard deviation.
[b] Relative error.

Recovery

Recovery experiments are also performed in order to demonstrate method accuracy. For recovery study QC samples were spiked with three different levels (low, middle and high) of valproate and subjected to the proposed method. **Table 3** shows the relative recoveries (expressed as RR %) data obtained during method validation. The calculated RRs were within the range of 93–113%; this demonstrates the suitability of the sample preparation method for the analysis of the examined compounds in plasma and urine samples.

Table 3. Relative recoveries obtained by the developed method in human plasma and urine samples spiked at different concentrations.

Sample	Nominal concentration (µg mL^{-1})	Found concentration (µg mL^{-1}) ± SD[a]	Relative recovery (RR%) ± SD
Plasma	0.5	0.49 ± 0.01	98 ± 2
	50	51 ± 0.4	102 ± 0.8
		505 ± 3	101 ± 0.5
Urine	0.1	0.1 ± 0.005	94 ± 5
	10	9 ± 0.1	93 ± 1.5
	200	200 ± 1	100 ± 1

[a] Standard deviation (n=3).

Specificity and selectivity

Specificity of the developed method was investigated by analyzing batches of blank samples and no significant interferences were observed at the retention times of the analytes from samples matrix. The selectivity of the method for the selected drug was also tested by analysis of some other co-administered AEDs (e.g., lamotrigine, phenobarbital, diazepam, clonazepam, and phenytoin) and most commonly used drugs such as oxazepam, cetirizine, naproxen, acetaminophen, and codeine. Selectivity tests were performed using therapeutic amount of each drug. Presence of non-volatile and basic drugs presents no problem due to the very different characteristics of the AEDs (boiling point, pK_a and volatility). These drugs show no

interference with our developed method because basic drugs are protonated in acidic medium, thus this form is poorly extracted and in most of cases, due to the non-volatile nature of the above mentioned drugs chromatography without prior derivatization is impossible.

Analyte stability

The stability of valproate was assessed by analyzing triplicate QC samples, exposed to different storage conditions including room temperature (25 ± 2.0 °C) for 12 h and three freeze–thaw cycles. No significant degradation of valproate was observed under various storage conditions (Table 4).

Table 4. Stability data for sodium valproate in human plasma and urine samples.

		Room temperature stability			Freeze–thaw stability		
Sample	Nominal concentration (μg mL^{-1}) (n = 3)	Found concentration (μg mL^{-1}) ± SD	Accuracy (RE %)	Recovery (%) ± SD	Found concentration (μg mL^{-1}) ± SD	Accuracy (RE %)	Recovery (%) ± SD
Plasma	*0.5*	*0.51* ± 0.04	*2.5*	*102* ± 7	*0.53* ± 0.02	*5.7*	*106* ± 5
	50	*51* ± 0.7	*2.8*	*103* ± 1	*51* ± 1	*2.7*	*103* ± 2
	500	*510* ± 6	*2.1*	*102* ± 1	*511* ± 5	*2.2*	*102* ± 1
Urine	*0.1*	*0.1* ± 0.01	*-11.1*	*89* ± 9	*0.1* ± 0.01	*-1.7*	*98* ± 9
	10	*9* ± 0.1	*-7.0*	*93* ± 1	*9.6* ± 0.4	*-3.8*	*96* ± 3.5
	200	*199* ± 2	*-0.6*	*99* ± 1	*201* ± 2	*0.3*	*100* ± 1

Robustness

Robustness of the method was evaluated by different volumes of the extraction solvent (60, 62, and 64 μL), various pHs (1.9, 2.0, and 2.1), and different NaCl concentrations (7, 7.5, and 8%, *w/v*). The obtained results were comparable with each other and the differences among them were not significant (Table 5).

Table 5. Evaluation of method robustness for the extraction and analysis of sodium valproate in the spiked human plasma and urine samples with LLME-GC-FID method.

Sample	Level	Nominal concentration (μg mL^{-1})	Found concentration (μg mL^{-1}) ± SD (n = 3)	Accuracy (RE %)	Relative recovery (%) ± SD
Plasma	1	50	50 ± 1	0.7	101 ± 3
	2	50	51 ± 0.4	1.5	102 ± 1
	3	50	51 ± 2	2.0	102 ± 3
Urine	1	10	9.9 ± 0.2	-1.4	99 ± 2
	2	10	9 ± 0.1	-7.0	93 ± 1
	3	10	9.8 ± 0.4	-2.0	98 ± 4

Level 1: pH = 1.9, extraction solvent volume: 60 μL, NaCl concentration: 7 %, *w/v*.
Level 2: pH = 2.0, extraction solvent volume: 62 μL, NaCl concentration: 7.5 %, *w/v*.
Level 3: pH = 2.1, extraction solvent volume: 64 μL, NaCl concentration: 8 %, *w/v*.

Application to real samples

The developed and validated LLME-GC-FID methods have been applied for the analysis of plasma and urine samples of 16 patients under treatment with sodium valporate. All patients gave their written consent, then blood and urine samples were collected from the patients after oral administration of the drug. The samples were analyzed three times and the drug concentration was calculated using the calibration curve. The results, along with some details of the patients, are presented in **Table 6**. GC-FID chromatograms of the spiked plasma and urine samples as well as one patient sample receiving sodium valproate are shown in Figures 3-6. Chromatograms (a) and (c) in the figures belong to the drug-free samples and standard solution (500 mg L^{-1}) prepared in chloroform, respectively. As can be seen, no interference peaks were observed while analyzing this drug in drug-free plasma and urine samples, which indicate that the method is suitable for clinical studies. In addition, none of the co-administered drugs were appeared in the chromatogram revealing the selectivity of the extraction and/or quantification method toward valproate.

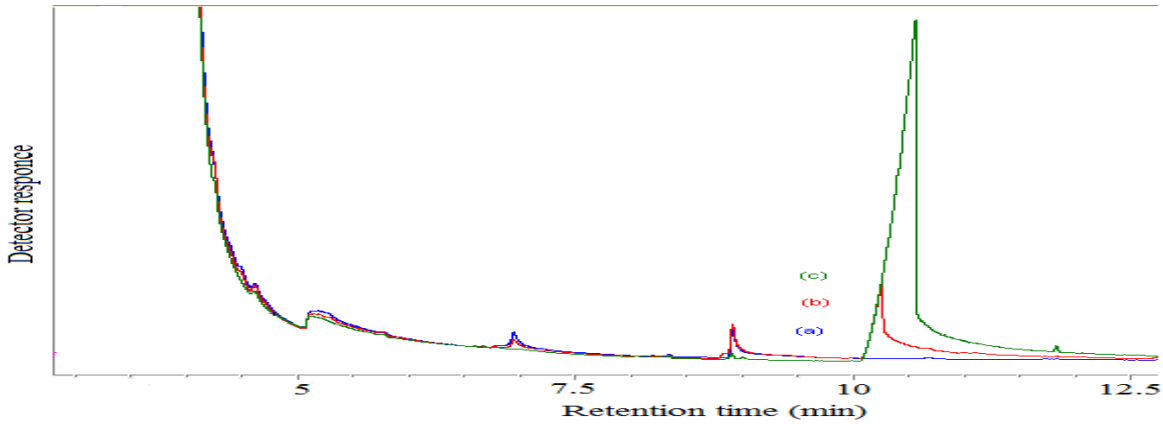

Fig. 3. GC-FID chromatograms of: (a) drug-free plasma, (b) plasma spiked with 10 µg mL^{-1} of sodium valproate, and (c) standard solution (500 mg L^{-1}) prepared in chloroform. Chromatogram (c) was obtained by direct injection whereas in the cases of two other chromatograms the proposed LLME method was carried out on the samples and 1 µL of the sedimented phase was injected into the separation system.

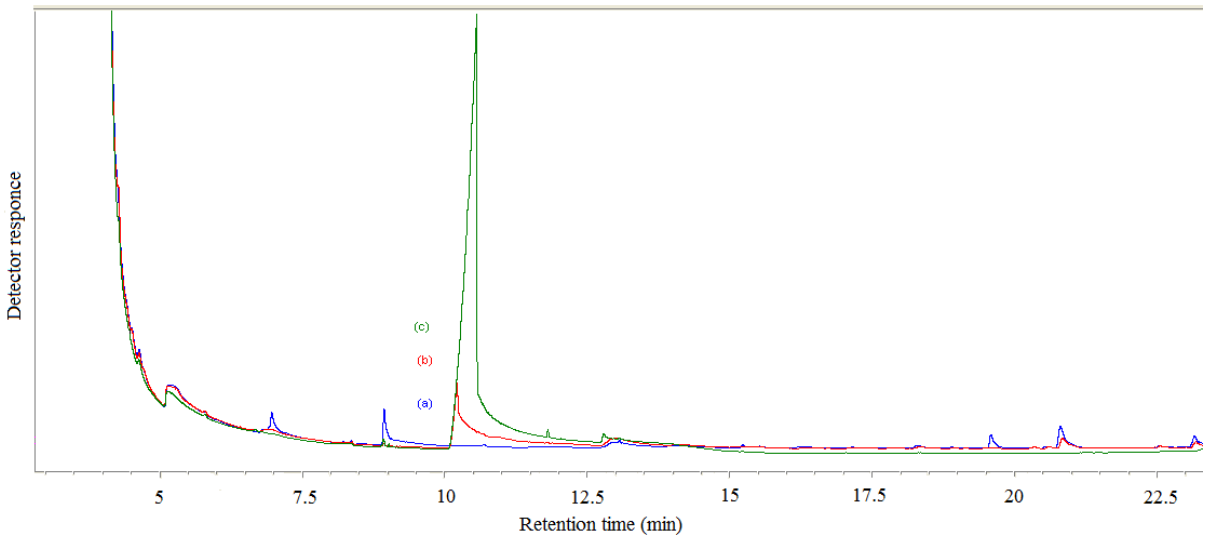

Fig. 4. Typical GC-FID chromatograms of (a) drug-free plasma sample, (b) plasma sample from patient with epilepsy, and (c) standard solution (500 mg L^{-1}) prepared in chloroform. Chromatogram (c) was obtained by direct injection whereas in the cases of two other chromatograms the proposed method was carried out on the samples and 1 µL of the sedimented phase was injected into the separation system.

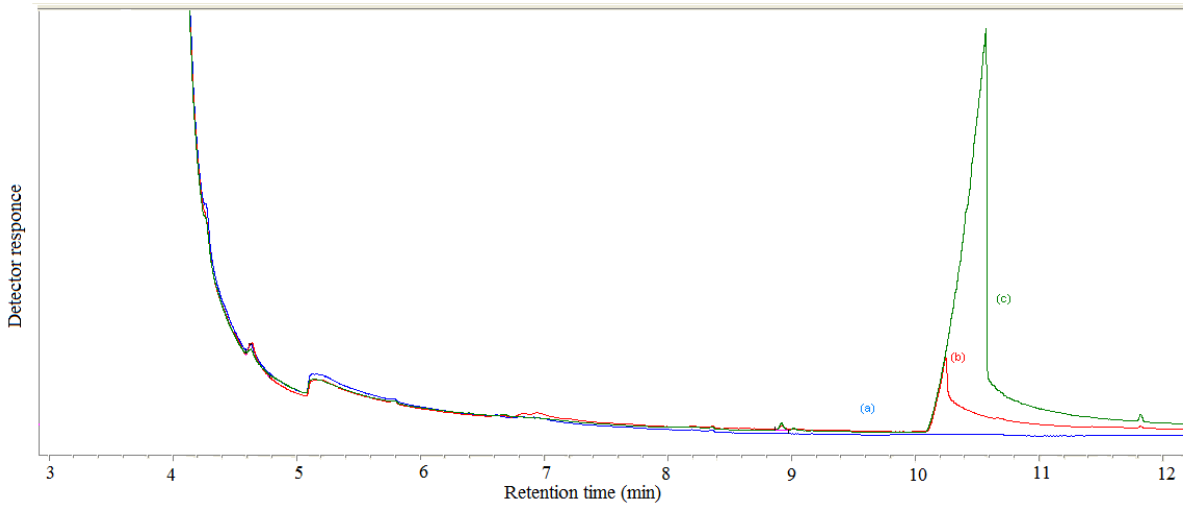

Fig. 5. GC-FID chromatograms of: (a) drug-free urine, (b) urine spiked with 2 µg mL^{-1} of sodium valproate, and (c) standard solution (500 mg L^{-1}) prepared in chloroform. Chromatogram (c) was obtained by direct injection whereas in the cases of other chromatograms the proposed LLME method was carried out on the samples and 1 µL of the sedimented phase was injected into the separation system.

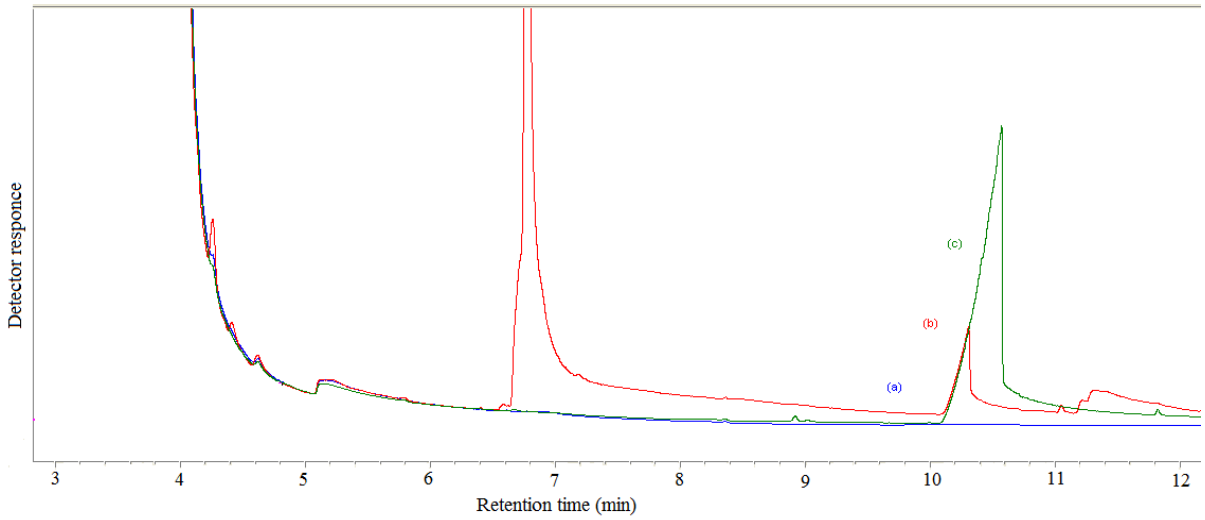

Fig. 6. Typical GC-FID chromatograms of (a) drug-free urine sample, (b) urine sample from patient with epilepsy, and (c) standard solution (500 mg L^{-1}) prepared in chloroform. Chromatogram (c) was obtained by direct injection whereas in the cases of two other chromatograms the proposed method was carried out on the samples and 1 µL of the sedimented phase was injected into the separation system.

Table 6. Determination of valproate in patients' samples by the proposed method (the results are given as mean results, n = 3).

No.	Gender	Age (year)	sodium valproate daily dosage (mg)	Duration of drug intake (M)	Co-administered drug	Concentration (µg mL^{-1}) ± SD	
						plasma	urine
1	M	47	400	5	Propranolol, Nortriptyline	12.8 ± 0.7	1.3 ± 0.1
2	F	53	400	180	Fluoxetine, Citalopram, Clonazepam, Nortriptyline, Propranolol, Imipramine, Omeprazole	12.9 ± 0.4	2.7 ± 0.2
3	F	52	250	10	Fluoxetine, Propranolol,Folic acid, Ferrous sulfate, Diclofenac	13.8 ± 0.7	3.4 ± 0.2
4	F	42	100	168	Citrizine, Calcium, Vitamin D	12.7 ± 0.5	5.2 ± 0.2
5	M	34	500	6	Warfarin, Chlordiazepoxide	19.9 ± 0.6	2.9 ± 0.1
6	F	14	200	72	Clonazepam	9.9 ± 0.9	0.5 ± 0.04
7	M	12	200	2	Calcium, Vitamin D	14.5 ± 0.6	3 ± 0.1
8	F	28	400	132	Calcium, Vitamin D, Clonazepam	12.4 ± 0.3	2.4 ± 0.1
9	F	15	400	3	Clonazepam, Fluoxetine	21.6 ± 0.2	3.3 ± 0.1
10	F	60	400	36	Acetylsalicylic acid	17.0 ± 0.4	1.2 ± 0.1
11	F	30	400	9	Fluoxetine, Nortriptyline, Trifluoperazine	13.6 ± 0.3	6.7 ± 0.1
12	M	40	500	17	Phenobarbital, Clonazepam	12 ± 0.5	2 ± 0.1
13	F	61	200	168	Atorvastatin, Sildenafil, Spironolactone, Busentan, Digoxin, Warfarin, Furosemide	14.6 ± 0.5	4 ± 0.1
14	F	12	600	2	Desmopressin	19.8 ± 0.6	5.9 ± 0.2
15	F	58	400	2	Fluoxetine, Clonazepam, Propranolol, Ergotamine	18.2 ± 0.7	0.6 ± 0.05
16	F	26	500	6	Lamotrigine	34.7 ± 0.4	5.4 ± 0.2

Comparison of the proposed method with other methods

Table 7 summarizes the values of LOD, LOQ, LR, and r^2 of some analytical methods along with the proposed method for the extraction and determination of the selected analyte in different samples. In comparison with other methods, the proposed method provides wider LRs. LODs for the proposed method are lower than or comparable with those of the mentioned methods except solid phase extraction–liquid chromatography-tandem mass spectrometry (SPE–LC–MS/MS). It should be noted that in this method, a high sensitive detection system (mass spectrometry) was used which is naturally more sensitive than FID. Low consumption of sample is another advantage of the method as compared with the others in screening of the interested analyte in the samples. It can be concluded that the presented LLME–GC–FID method is a sensitive, rapid and repeatable method that can be used for the preconcentration and determination of the studied analyte in biological samples.

Table 7. Comparison of the presented method with other methods used in preconcentration and determination of sodium valproate in biological fluids.

Sample	LOD[a)] (μg mL^{-1})	LOQ[b)] (μg mL^{-1})	LR[c)] (μg mL^{-1})	r ^{2}[d)]	Method validation	Method	Ref.
Human plasma	-	5	5-200	-	Yes	LLE-HPLC-UV[e)]	6
Human plasma	0.03	2.03	2.03-152.25	0.997	Yes	SPE–LC–MS/MS[f)]	12
Human plasma	0.08	0.24	0.4-300	0.9992	No	DLLME-CE-CCD[g)]	16
Serum	-	8	25-400	0.9992	No	LLE-GC-FID[h)]	29
		25	25-400	0.9998		LLE-HPLC-UV	
Human plasma	0.15	0.45	0.45-100	0.998	No selectivity test	LLE-GC-FID	30
Human serum	0.8	-	2-20	0.96>	No	HS-LPME-GC-FID[i)]	31
Human plasma	3.2	6	6-140	0.998>	Yes	DLLME- GC-FID	37
Human plasma	0.22	0.73	0.5-500	0.9999	Yes	DLLME-GC-FID	This work
Urine	0.05	0.16	0.1-200	0.9996		AALLME-GC-FID[j)]	

[a] Limit of detection.
[b] Limit of quantification.
[c] Linear range.
[d] Square of correlation coefficient.
[e] Liquid–liquid extraction– high performance liquid chromatography-ultraviolet detection.
[f] Solid phase extraction –liquid chromatography–tandem mass spectrometry.
[g] Dispersive liquid–liquid microextraction –capillary electrophoresis –contactless conductivity detection.
[h] Liquid–liquid extraction– gas chromatography–flame ionization detection.
[i] Headspace– liquid phase microextraction– gas chromatography-flame ionization detection.
[j] Air-assisted liquid–liquid microextraction –gas chromatography –flame ionization detection.

Conclusion

Microextraction methods based on LLME have been reported for the extraction and preconcentration of sodium valproate from biological samples followed by GC–FID determination. The developed method has numerous advantages such as rapidness, simplicity, and better repeatability. In comparison with the conventional LLE procedures, the main advantage of this method is low consumption of extraction solvent. Some disadvantages associated with DLLME methods including multi-step pretreatment and also relatively higher number of parameters to be optimized are the main limitations of the proposed method. The obtained results revealed that the proposed method is a suitable analytical method for routine applications in biomedical analysis laboratories.

Conflict of interest

Authors certify that there is no actual or potential conflict of interest in relation to this article.

References

[1] Jabbaribar F, Mortazavi AR, Jouyban A. Analytical methods for determination of antiepileptic drugs. Curr Pharm Anal. 2009;5:277-345.

[2] Rani S, Malik AK, Kaur R, Kaur R. A review for the analysis of antidepressant, antiepileptic and quinolone type drugs in pharmaceuticals and environmental Samples. Crit Rev Anal Chem. 2016;46:424-442.

[3] Kazemipour M, Fakhari I, Ansari M. Gabapentin determination in human plasma and capsule by coupling of solid phase extraction, derivatization reaction, and UV-Vis spectroscopy. Iran J Pharm Res. 2013;12:247-253.

[4] Qu L, Fan Y, Wang W, Ma K, Yin Z. Development, validation and clinical application of an online-SPE-LC-HRMS/MS for simultaneous quantification of phenobarbital, phenytoin, carbamazepine, and its active metabolite carbamazepine 10,11-epoxide. Talanta. 2016;158:77-88.

[5] O`Neil, MJ., The Merck Index. An encyclopedia of chemicals, drugs and biologicals. 13th ed., Raway, New Jersey, 2001.

[6] Chen Zh, Wang X, Wang H, Chen Sh, Zhou L, Li J, Shu W, Zhou J, Fang Z, Zhang Y, Huang M. Simultaneous determination of valproic acid and 2-propyl-4-pentenoic acid for the prediction of clinical adverse effects in Chinese patients with epilepsy. Seizure. 2012;21:110–117.

[7] Begley CE, Famular MI, Anneger JFS, Lairson DR, Reynolds TF, Coan S, Dubisky S, Newmark ME, Leibson C, So EL, Rocca WA. The cost of epilepsy in the United State: An estimate from population-based clinical and survey Data. Epilepsia. 2000;41:342-51.

[8] DeVane C. Pharmacokinetics, drug interactions, and tolerability of valproate. Psychopharmacol Bull. 2003;37:25-42.

[9] Vajda FJ, Drummer OH, Morris, McNeil JJ, Bladin PF. Gas chromatographic measurement of plasma levels of sodium valproate: tentative therapeutic range of a new anticonvulsant in the treatment of refractory epileptics. Clin Exp Pharmacol Physiol. 1978;5:67–73.

[10] Covanis A, Gupta AK, Jeavons PM. Sodium valproate: monotherapy and polytherapy. Epilepsia. 1982;23:693–720.

[11] Souza PRSE, de Carvalho JM, Albert ALM, Moreira JC, Leandro KCh. Development and validation of a method for the determination of valproic acid in pharmaceutical formulations by high performance liquid chromatography with diode array detection (HPLC-DAD). Vigilância Sanitáriaem Debate. 2013;1:52-58.

[12] Gao Sh, Miao H, Tao x, Jiang B, XiaoY, Cai F, Yun Y, Li J, Chen W. LC–MS/MS method for simultaneous determination of valproic acid and major metabolites in human plasma. J Chromatogr B. 2011;879:1939–1944.

[13] Levy RH, Shen DD. Valproic Acid: Absorption, Distribution, and Excretion. In Levy RH, Mattson RH, Meldrum BS (eds), Antiepileptic Drugs. 4th ed., Raven Press, New York, pp 605-619, 1995.

[14] Konig SA, Knolle J, Friedewald S, Koelfen W, Longin E, Lenz T, Hannak D. Effects of Valproic Acid, Carbamazepine, and Phenobarbitone on the Fatty Acid Composition of Erythrocyte Membranes in Children. Epilepsia. 2003;44:708-711.

[15] Pucci V, Mandrioli R, Raggi MA. Determination of valproic acid (2-propylpentanoic acid) in human plasma by capillary electrophoresis with indirect UV detection. Electrophoresis. 2003;24:2076-2083.

[16] Phama TTT, Seea HH, Morandd R, Krahenbuhld S, Hausera PC. Determination of free and total valproic acid in human plasma by capillary electrophoresis with contactless conductivity detection. J Chromatogr B. 2012; 907:74–78.

[17] Cheng H, Liu Z, Blum W, Byrd JC, Klisovic R, Grever MR, Marcucci G, Chan KK. Quantification of valproic acid and its metabolite 2-propyl-4-pentenoic acid in human plasma using HPLC–MS/MS. J Chromatogr B. 2007;850:206–12.

[18] Deepak SJ, Gunta S, Mallika S, Pranav S. A high throughput and selective method for the estimation of Valproic acid an antiepileptic drug in human plasma by tandem LC-MS/MS. Talanta. 2007;72:80-88.

[19] Tsukasa M, Masaharu N, Hiroyuki W, Susumu Y, Kenji S. Determination of valproic acid in serum by liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. Anal Sci. 2007;17:999-1001.

[20] Vlase L, Popa DS, Muntean D, Leucuta S. A New high-throughput LC-MS/MS assay for

therapeutic level monitoring of valproic acid in human plasma. Sci Pharm. 2008;76:663–671.
[21] Amini H, Javan M, Ahmadiani A. Development and validation of a sensitive assay of Valproic acid in human plasma by high-performance liquid chromatography without prior derivatization. J Chromatogr B. 2006;830:368-71.
[22] Kishore P, Rajani KV, Satyanarayana V, Krishna DR. HPLC determination of valproic acid in human serum. Phammazie. 2003;58:378-80.
[23] Lin MC, Kou HS, Chen CC, Wu SM, Wu HL. Simple and sensitive fluorimetric liquid chromatography method for the determination of valproic acid in plasma. J Chromatogr B. 2004;810:169-172.
[24] Gupta RK, Singh UK, Kumar S, Moothan B. Estimation of sodium valproate in tablet dosage form by RP-HPLC without prior derivatization: Application to dissolution studies. Int J Pharm Sci Drug Res. 2009;1:103-106.
[25] Yan Z, Zheng J, Yunqiu Y. Simultaneous determination of mycophenolic acid and Valproic acid based on derivatization by high-performance liquid chromatography with fluorescence detection. Biomed Chromatogr. 2006;20:319-326.
[26] Pravin S, Abdorreza M, Naader A. Determination of the valproic acid in human serum and pharmaceutical preparation by headspace liquid phase microextraction gas chromatography flame ionization detection without prior derivatization. J Chromatogr B. 2006;850:128-133.
[27] The United States Pharmacopeial Convention. The United States Pharmacopeia- The National Formulary. Asian Edition. Volum 3. Port City Pres, United States. pp 3442, 2007.
[28] Kang J, Park YS, Kim SH, Kim SH, Jun MY. Modern methods for analysis of antiepileptic drugs in the biological fluids for pharmacokinetics, bioequivalence and therapeutic drug monitoring. Korean J Physiol Pharmacol. 2011;15:67-81.
[29] Nazeri A, Jalali M, Aliasgharpour M, Khosravie F. Comparison of serum valproic acid determination through gas and high performance liquid chromatography methods. Health Scope. 2014;2(4):12085.
[30] Ahmadkhaniha R, Rastkari N, Kobarfard F, Pakdaman H, Ahmadkhaniha O, Kebriaeezadeh A. An improved GC method for rapid analysis of valproic acid in human plasma without derivatization. Iran J Pharm Sci. 2007;3:37-42.
[31] 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. J Chromatogr B. 2007, 850:128-133.
[32] Gopaul SV, Farrell K, Abbott FS. Gas chromatography/negative ion chemical ionization mass spectrometry and liquid chromatography/electrospray ionization tandem mass spectrometry quantitative profiling of N-acetylcysteine conjugates of valproic acid in urine: application in drug metabolism studies in humans. J Mass Spectrom. 2000;35:698-704.
[33] Anari MR, Burton RW, Gopaul S, Abbott FS. Metabolic profiling of valproic acid by cDNA-expressed human cytochrome P450 enzymes using negative-ion chemical ionization gas chromatography-mass spectrometry. J Chromatogr B. 2000;742:217-227.
[34] Nagai G, Ono S, Yasui-Furukori N, Nakamura A, Mihara K, Kondo T. Formulations of valproate alter valproate metabolism: a single oral dose kinetic study. Ther Drug Monit. 2009;31:592-596.
[35] Krogh M, Johansen K, Tønnesen F, Rasmussen KE. Solid-phase microextraction for the determination of the free concentration of valproic acid in human plasma by capillary gas chromatography. J Chromatogr B. 1995;673:299-305.
[36] Sadeghi R, Kobarfard F, Yazdanpanah H, Eslamizad S, Bayate M. Simultaneous determination of 13 priority polycyclic aromatic hydrocarbons in Tehran's tap water and water for injection samples using dispersive liquid-liquid micro extraction method and gas chromatography-mass spectrometry. Iran J Pharm Res. 2016; 15:475-481.
[37] 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. Iran J Basic Med Sci. 2015;18:979-988.
[38] Sadeghi R, Kobarfard F, Yazdanpanah H, Eslamizad S, Bayat M. Validation of an analytical method for determination of 13 priority polycyclic aromatic hydrocarbons in mineral water using dispersive liquid-liquid microextraction and GC-MS. Iran J Pharm Res. 2016;15:157-168.
[39] Parsaei P, Bahmaei M, Ghannadi A. Determination of some B vitamins in sour cherry juice using dispersive liquid-liquid microextraction followed by high-performance

liquid chromatography. Iran J Pharm Res. 2014;13:1435-1443.
[40] Helena P, Locija ZK. Solid-phase microextraction; Trends in Analytical Chemistry. Trends Anal Chem. 1999;18:272-282.
[41] Liu S, Dasgupta PK. A Renewable Gas Sampling Interface. Anal Chem. 1995;67:2042-2049.
[42] Rezaee M, Assadi Y, Hosseini MRM, Aghaee E, Ahmadi F, Berijani S. Determination of organic compounds in water using dispersive liquid–liquid microextraction. J Chromatogr A. 2006;1116:1-9.
[43] Regueiro J, Llompart M, Garcia-Jares C, Garcia-Monteagudo JC, Cela R. Ultrasound-assisted emulsification–microextraction of emergent contaminants and pesticides in environmental waters. J Chromatogr A. 2008;1190:27-38.
[44] Yiantzi E, Psillakis E, Tyrovola K, Kalogerakis N. Vortex-assisted liquid–liquid microextraction of octylphenol, nonylphenol and bisphenol-A. Talanta. 2010;80:2057-2062.
[45] Farajzadeh MA, AfsharMogaddam MR. 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;728:31-38.
[46] FDA Guidelines (2001) US Department of Health and Human Services, Center for Drug Evaluation and Research, Center for Veterinary Medicine. Guidance for Industry Bioanalytical Method Validation.

