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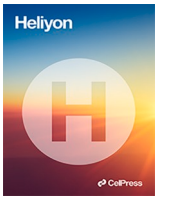

Research article

Development of UPLC–MS/MS method for the simultaneous quantification of valproic acid and phenytoin in human plasma and application to study pharmacokinetic interaction in epilepsy patients

Sil Thanh Nguyen [a], Duy Nguyen Ho [a], Thi Anh Huynh Huynh [b], Huyen Thu Thi Nguyen [c], Ngan Kim Thi Ly [d], Minh Van Le [d], Tho Vinh Minh Chau Do [e,*]

[a] *Faculty of Pharmacy, Can Tho University of Medicine and Pharmacy, Can Tho, 900000, Viet Nam*
[b] *Department of Chemistry, College of Natural Sciences, Can Tho University, Can Tho, 900000, Viet Nam*
[c] *Institute of Drug Quality Control Ho Chi Minh City, Ho Chi Minh City, 700000, Viet Nam*
[d] *Faculty of Medicine, Can Tho University of Medicine and Pharmacy, Can Tho, 900000, Viet Nam*
[e] *Department of Analytical Chemistry - Toxicology - Drug Quality Control, Faculty of Pharmacy, Can Tho University of Medicine and Pharmacy, Can Tho, 900000, Viet Nam*

A R T I C L E I N F O

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A B S T R A C T

Valproic acid and phenytoin are two prevalent antiepileptic medications known for their narrow indices and propensity for cardiovascular and respiratory system toxicity. Therefore, therapeutic drug monitoring (TDM) of valproic acid (VAL) and phenytoin (PHE) concentrations in patient plasma is extremely beneficial for improving clinical choices, avoiding adverse reactions, and optimizing treatment for individual patients. In this study, a rapid and sensitive ultra-performance liquid chromatographic tandem mass spectrometer (UPLC-MS/MS) method was developed and validated for the simultaneous quantitative determination of valproic acid (VAL) and phenytoin (PHE) in human plasma. Negative electron spray ionization (ESI-) mode with selective ion recording (SIR) was employed to determine the transitions of *m/z* 142.98 and *m/z* 250.93 for VAL and PHE, respectively. The internal standard (IS) betamethasone (BETA) was ionized using positive electron spray ionization (ESI+) and detected by multi-reaction monitoring (MRM) mode to obtain precursor ions and specific fragment ions for quantification, and the MRM transition was chosen to be *m/z* 393.17 → 355.16. The separation was performed using a Phenomenex Synergi Hydro-RP (4 μm, 250 × 4.6 mm, I.D.) with an isocratic mobile phase consisting of acetonitrile – water (75:25, v/v) at a flow rate of 0.8 mL/min. The column temperature was maintained at 25 °C. The lower limit of quantification of VAL and PHE was 3.6 μg/mL and 0.72 μg/mL, respectively, which resulted in a recovery of more than 85 % for most analytes. According to US-FDA bioanalytical technique validation, the specificity, intra- and inter-day precision and accuracy, matrix effect, carryover, dilution, and stability of all analytes were within acceptable ranges. This analytical method was successful in evaluating the levels of valproic acid and phenytoin in human plasma from epileptic patients.

* Corresponding author.
E-mail address: dcmvtho@ctump.edu.vn (T.V. Minh Chau Do).

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1. Introduction

About 50 million individuals worldwide suffer from epilepsy, a chronic, noncommunicable brain condition [1,2]. Some substances such as phenytoin, valproic acid, phenobarbital, carbamazepine, and lamotrigine, are used alone or in combination in the treatment of epilepsy from mild to severe [3]. The choice of drugs is appropriate for the patient's condition, and the patient's economic status, improving the patient's quality of life, and minimizing the negative side effects of the drug are of utmost importance.

In antiepileptic drug (AED) therapy, VAL and PHE are among the first-line agents [4,5]. VAL or sodium valproate is an antiepileptic drug for many types of seizures, that is dissociated into the valproate ion in the gastrointestinal tract. The antiepileptic effect of valproate is probably through the inhibitory neurotransmitter gamma-aminobutyric acid (GABA). Peak plasma concentrations of VAL are reached 1–4 h after a single dose of VAL. Meanwhile, PHE is a hydantoin derivative and a first-generation anti-convulsant medication that treats generalized tonic-clonic seizures, complex partial seizures, and status epilepticus without compromising neurological function appreciably. Phenytoin acts by blocking voltage-dependent membrane sodium channels that cause the action potential to rise. Oral bioavailability is between 80 and 95 %, with peak plasma concentrations reaching after 1.5–3 h. However, the correlation between the daily dose, plasma concentrations, and therapeutic effects of VAL and PHE has not been established. VAL and PHE have a narrow therapeutic index, only considering the therapeutic range at valproic acid plasma concentrations of 50–100 μg/mL and phenytoin plasma concentrations of 10–20 μg/mL [6–8].

Many quantitative research studies on valproic acid and phenytoin have been reported using a variety of techniques such as chemiluminescence immunoassay (CLIA) [9], UV or PDA detector coupled liquid chromatography [10], MS or MS/MS probe coupled liquid chromatography [11,12], gas chromatography coupled to mass spectrometry (GC-MS) [13,14], and others. Due to very low plasma drug concentrations, ultraviolet detection's sensitivity is insufficient, while fluorescence detection offers a reasonably high sensitivity, the laborious and time-consuming derivatization and extraction technique is a limitation, especially in the face of high throughput demands. A method with high sensitivity and specificity such as UPLC with an MS detector has revolutionized the field of bioanalysis.

To the best of our knowledge, no research has been done till date on using the UPLC-MS/MS technology to simultaneously quantify epilepsy medications like valproic acid and phenytoin. Therefore, the aim of this study was to develop and validate a selective, sensitive, and trustworthy UPLC-MS/MS method for the simultaneous detection of VAL and PHE in human plasma (Fig. 1) in order to provide a strong tool for prospective bioequivalence studies as well as therapeutic drug monitoring. In addition to this, these are useful for drug-drug interaction (DDI) investigations.

2. Materials and method

2.1. Chemicals and reagents

Reference standards of sodium valproate (98.0 %), phenytoin (99.7 %), and betamethasone (98.5 %) were provided by the Institute of Drug Quality Control Ho Chi Minh city (Vietnam). LC-MS grade methanol, acetonitrile, and water were purchased from Merck (Darmstadt, Germany). All solvents or chemicals for sample preparation such as formic acid, acetic acid, methyl *tert*-butyl ether, and ethyl acetate met analytical standards.

Blank human plasma from healthy people (with heparin as an anticoagulant) was supplied by Can Tho Hematology Blood Transfusion Hospital (Can Tho, Vietnam), and six batches were stored at −40 °C. Application samples were provided by Can Tho Central Genel Hospital (Can Tho, Vietnam), and stored at −40 °C until the analysis.

2.2. Instruments and analytical conditions

The UPLC-MS/MS system included an Ultra Performance Liquid Chromatography (WATERS ACQUITY UPLC H-Class) and a triple-quadrupole mass spectrometer (Xevo-TQD) equipped with an electrospray ionization (ESI) probe was used to quantify simultaneous VAL and PHE in the plasma of Vietnamese epilepsy patients. All UPLC and MS/MS parameters were managed by the Masslynx™ version 4.2 software (Waters Corporation, Milford, MA, USA).

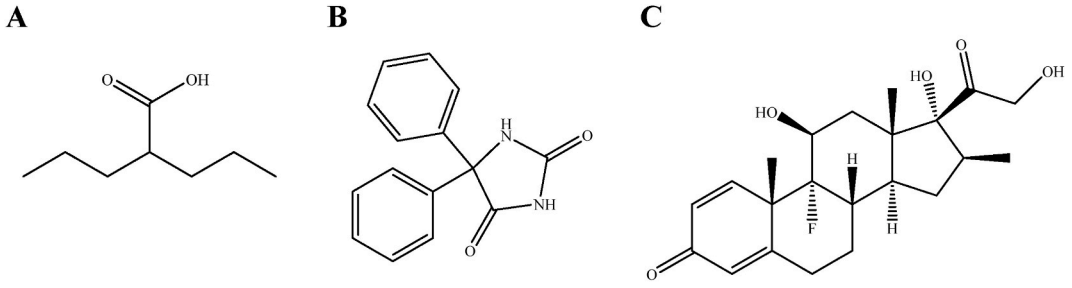

Fig. 1. Chemical structures of VAL (A), PHE (B), BETA (C) as internal standard.

The MS/MS detections were performed with negative ion mode (ESI-) for VAL and PHE, in addition to selective ion recording (SIR); positive ion mode (ESI+) for IS (BETA), as well as multi-reaction monitoring (MRM). The capillary voltage, source temperature, desolvation temperature, and desolvation gas flow rate were optimized and set at 4.0 kV, 150 °C, 500 °C, and 1000 L/Hr, respectively. The quantification was carried out with the SIR mode of VAL and PHE at *m/z* 142.98 and 250.93, respectively, and with the MRM mode of IS at *m/z* 393.17 → 147.08 and 355.16. Table 1 shows all the optimized parameter conditions for detected components.

2.3. Preparation of stock and working solutions, calibrations standards, and quality control (QC) samples

The standard stock solutions of VAL, PHE, and IS were separately dissolved in a 50 mL volumetric flask with the appropriate soluble solvent (acetonitrile for VAL and methanol for PHE and IS) to achieve a concentration of 500 μg/mL for each analyte. Subsequently, a combined standard solution containing VAL, PHE, and IS at a concentration of 200 ng/mL was prepared and directly injected into the mass spectrometry instrument for the assessment of chromatographic and mass spectrometry conditions.

Ten calibration standard solutions were made by spiking standard working solutions with human blank plasma in the range of 3.6–144 μg/mL, and 0.72–28.8 μg/mL, respectively. The quality control (QC) samples were prepared from blank plasma at the lower limit (LLOQ), low (LQC), medium (MQC), high (HQC), and ultra limit (ULOQ) concentrations of 3.6 μg/mL, 10.8 μg/mL, 60 μg/mL, 108 μg/mL, 144 μg/mL for VAL, 0.72 μg/mL, 2.16 μg/mL, 12 μg/mL, 21.6 μg/mL, 28.8 μg/mL for PHE, respectively. All solutions were stored at −40 °C and must be left at room temperature until analysis.

2.4. Sample preparation

After thawing, a 200 μL aliquot of plasma sample was pipetted into a microcentrifuge tube, 200 μL IS working solution, 3.35 mL acetonitrile, and 250 μL acid formic (0.06 %, v/v) were added and vortex-mixed for 60 s. The mixture was centrifuged at 4427×*g* for 10 min. The supernatant organic phase was separated and 500 μL of this solvent was transferred to another tube, with the addition of 2.5 mL of a mixture of acetonitrile - water (75:25, v/v) and finally filtered through a 0.22 μm membrane to inject into the UPLC-MS/MS system.

2.5. Human sample collection

A total of 105 samples were obtained from epilepsy patients aged 16 years and older at Can Tho Central General Hospital (Can Tho, Vietnam). Among these patients, 84 were on VAL monotherapy at a dose of 500 mg administered twice daily, while 21 received a combination therapy of VAL alongside PHE for a duration exceeding one month. After one month of therapy, venous blood samples (2 mL) were collected for analysis immediately before taking morning drugs and stored at −40 °C. The proportion of epileptic women was higher than men, accounting for 52.5 %. While the average weight was approximately 54.46 kg, and the majority of patients resided in rural areas, it was 73.8 % compared to 26.2 % in urban areas. The protocol of this study was approved by the Human Investigation Ethics Committee of the Can Tho University of Medicine and Pharmacy, Vietnam.

2.6. Assay validation

The approach was validated in compliance with the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) bioanalytical method guidelines, including system suitability, specificity, the lower limit of quantitation, linearity and calibration curve, inter- and intra-day accuracy and precision, extraction recoveries, stability experiments, matrix effect, the effect of residual sample, and dilution integrity [15,16].

Table 1
The optimized mass spectrometry conditions of VAL, PHE and IS.

Parameter	Analyte		
	VAL	PHE	IS
Ion type	ESI(−)	ESI(−)	ESI(+)
Ion selective technique	SIR	SIR	MRM
Capillary voltage (kV)	4.0	4.0	4.0
Source temperature (°C)	500	500	500
Desolvation flow rate (L/Hr)	1000	1000	1000
Precursor ion (*m/z*)	142.98	250.93	393.17
Cone voltage (V)	30	40	28
Fragments (*m/z*)	–	–	355.16[a]
	–	–	147.08[b]
Collision energy	–	–	18
Dwell time (mms)	300	300	300

[a] Quantifier daughter fragment.
[b] Qualifier daughter fragment.

3. Results and discussion

3.1. Method development

Utilizing the auto-tune functionality within the Masslynx™ 4.2 software of the Waters system enables automated exploration of the most favorable settings pertaining to the mass spectrum. Following this, fine-tuning and confirmation of these parameters are performed using the manual-tune tool. During development, the sensitivity of full-scan in negative ion mode for VAL and PHE was higher than that in positive ion mode. The optimum mass spectrometry parameters are shown in Table 1, and the mass spectrum plot is shown in Fig. 2. VAL and PHE are small molecules, so very low or high collision energies did not produce sufficiently abundant and characteristic fragment ions. Therefore, the detection of ions was performed in SIR mode by selecting precursor ions without collision. On the other hand, BETA with its large molecular structure provided stable and specific product ion fragment signals. The identified mass spectrometry conditions in this study have been corroborated to be consistent with data sourced from reputable references and established findings from prior published research [11,12,17].

The efficiency of UPLC in separating compounds relies on various influential factors. The selection of the stationary phase in the chromatographic column is crucial, with its diverse compositions and properties determining both selectivity and resolution. Likewise, the mobile phase composition, including solvent type, pH, and gradient profile, serves as a fundamental influencer, directly shaping the retention of analytes and their elution patterns. In fact, because of having opposite properties, adding a pH adjuster to the mobile phase solvent had a significant effect on the peak shape and signal of VAL and PHE. The acidic adjuster produced a symmetric peak of VAL while causing a tailing peak of PHE. In contrast, the base was the reason VAL peak in poor shape. The choice of acetonitrile – water (75:25, v/v) without a pH adjuster as the mobile phase produced symmetrically shaped peaks, high signals, and short analysis times to ensure efficient application on TDM process. Eventually, chromatographic separations were performed on a Phenomenex Synergi Hydro-RP column (4 μm, 250 × 4.6 mm, I.D.), operated at 25 °C with isocratic elution at a flow rate of 0.8 mL/min. The mobile phase consisted of acetonitrile (A) and water (B) in a 75:25 (v/v) ratio. The injection volume was 10 μL and the analysis time was 6.0 min per sample. After 10 injections, rinse the column with a mixture of tetrahydrofuran-methanol-10 % acetic acid (5:4:1, v/v/v) for 30 min.

For the development of analytical methods, particularly in the analysis of biological fluids, establishing a sample processing

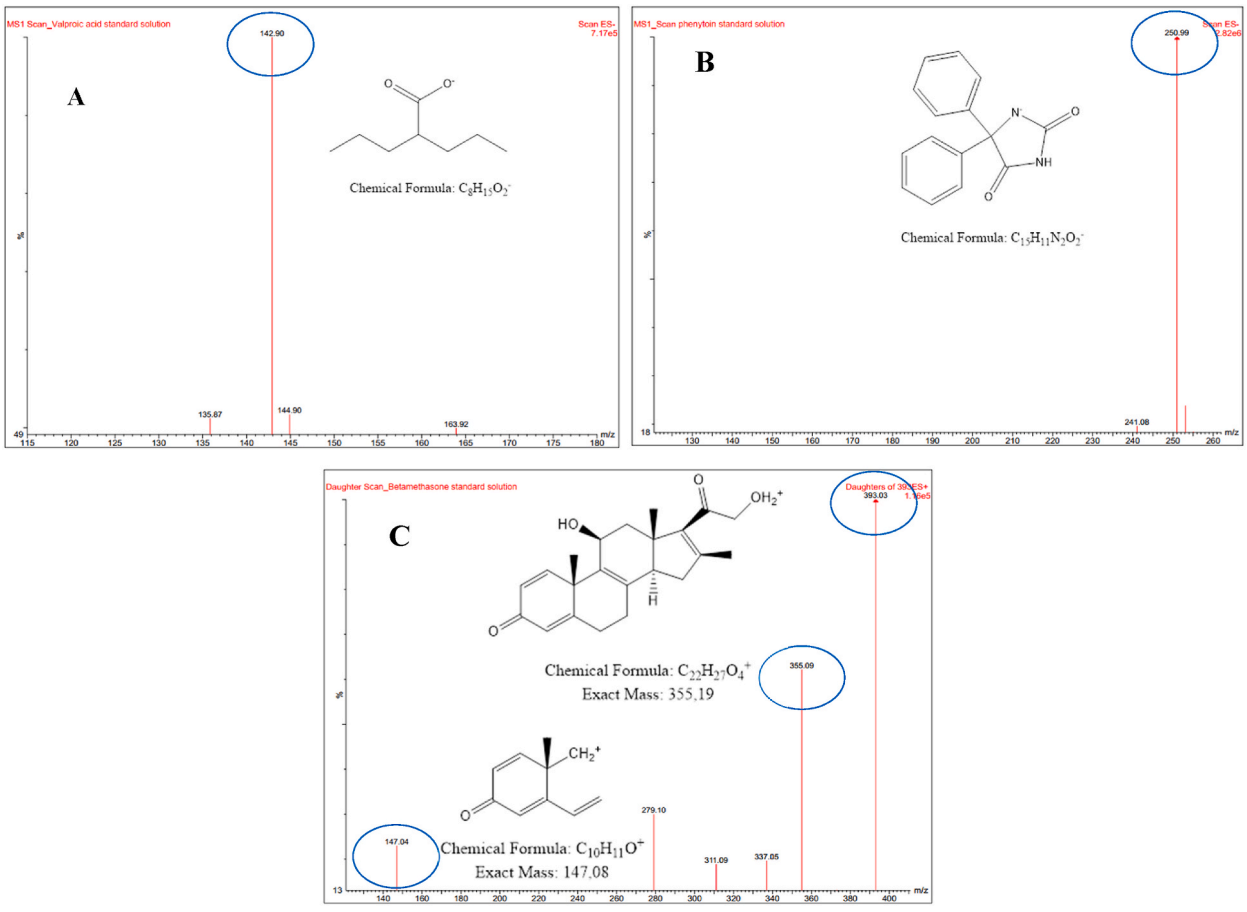

Fig. 2. Mass spectrum in SIR of VAL (A), PHE (B) and in MRM of BETA (C).

method that is simple, time-saving, minimizes sample loss, and ensures a clean sample background is an essential objective. Although the efficiency of extracting VAL and PHE from plasma by solid-phase extraction is high, it is expensive and time-consuming. Two methods of protein precipitation and liquid-liquid extraction were investigated to optimize the sample clean-up method to reduce matrix effects, increase extraction efficiency, and prolong the life of the analytical column [18,19]. In this study, the protein precipitation method was applied as a simple, cheap, fast, and eco-friendly method. Extraction efficiency during protein precipitation was tested using acetonitrile, methanol, and mixtures. To improve the recovery rate, different concentrations of formic acid that give the analyte an acidic molecular state were added to the plasma after adding acetonitrile. Formic acid impacts the pH of the environment, contributing to alterations in the sample's polarity and separation [18]. Adjusting the concentration and pH of formic acid may be necessary to maintain and enhance the shape of the peak, ensuring the quality and accuracy of the analytical results. The results showed that the recovery rate in the 0.06 % formic acid group was higher than other groups in this test (87.0–100.3 %). Finally, acetonitrile was used as the extraction solution before adding 250 μL of 0.06 % formic acid to the plasma.

3.2. Method validation

3.2.1. System suitability

The test is designed to confirm that the measuring system and the analytical processes connected with the analytical method can provide accurate and reliable findings when used. Repeatedly inject six times a simulated sample that contains a mixture of analytes and IS in concentration at MQC.

The results showed that the parameters of retention time, peak area, peak area ratio, and retention time ratio of analytes and IS when injected six times have a relative standard deviation (RSD) < 5 %.

3.2.2. Selectivity, specification

Selectivity demonstrates that the analytes in mixtures or matrixes could be measured without interference from other components of endogenous and related metabolites, degradation products, or concomitant drugs under investigation. The response of the blank plasma samples had to be within acceptable limits (≤ 20 % for analytes and ≤ 5 % for IS relative to their signals in LLOQ samples).

The chromatogram of the sample at LLOQ revealed peaks of VAL (4.47 min), PHE (3.10 min), and BETA (3.15 min). The retention time ratios of VAL/IS and PHE/IS were 1.42 and 0.98, respectively. The blank plasma samples showed no peak at the retention times of VAL, PHE, and IS. Thus, the specificity and reliability of the method can be confirmed.

3.2.3. Lower limit of quantification (LLOQ)

The LLOQ must be capable of determining the concentration of a sample obtained at a time comparable to 3–5 times the half-life, or 1/20-1/10 of the maximum analytes' concentration in the patient's blood. The lowest point of the calibration curve shall be considered as the lower limit of quantification (LLOQ) if the response is at least 5 times the response of the blank sample. The test was performed by injecting six QC samples at LLOQ concentrations.

The LLOQ of VAL and PHE in plasma was 3.60 μg/mL and 0.72 μg/mL, respectively, with acceptable accuracy within $\pm$ 20 % and precision (RSD%) $\leq$ 20 %.

3.2.4. Linearity of calibration curves

The calibration curves were described by the linear equation: $y = ax + b$, where y is the ratio of the analyte peaks and the corresponding IS peaks; x is the concentration of the analyte (μg/mL). Then, the regression coefficient, slope, and y-intercept of the resulting calibration curves were determined by least squared weighted regression ($1/x^2$).

The standard calibration curves for VAL and PHE in spiked human plasma were linear over the ranges 3.60–144 μg/mL, and 0.72–28.80 μg/mL, respectively, with correlation coefficients greater than 0.98 for both analytes. This linear range is compatible with their therapeutic range in human plasma and allows quantification at various sample periods as well as among medications used in different formulations. This is also the foundation for future bioequivalence evaluation research. The specific concentration ranges and individual equations for each analyte can be found in Table 2.

3.2.5. Assay precision and accuracy

Accuracy and precision were determined simultaneously on human plasma samples containing the analytes at four levels of quality control samples (LLOQ, LQC, MQC, and HQC). The intra- and inter-day precision and accuracy for the two analytes from the QC samples were summarized in Table 3. All parameters are within the allowable range. The chromatograms of these analytes and IS in quality control (QC) samples are shown in Fig. 3.

Table 2
Correlation coefficients, concentration range, and limit of detection of five anti-epilepsy drugs.

Analyte	Calibration equation	Correlation coefficient	Concentration range (μg/mL)	LLOQ (μg/mL)
VAL	Y = 5.01599*X – 4.92859	0.9954	3.60–144.0	3.60
PHE	Y = 14.5413*X + 15.3681	0.9977	0.72–28.80	0.72

X: the plasma concentration of each analyte (μg/mL).
Y: the peak area of each analyte to IS.

3.2.6. Recovery and matrix effect

The extraction recoveries were evaluated by comparing the spiked plasma samples at three concentrations (LQC, MQC, and HQC) with the standard sample of the same corresponding concentration. Prepare six samples at each concentration level. Perform chromatography on these samples. The recovery was calculated by comparing the peak areas of the pre-extraction spiked samples to those on the post-extraction spiked references.

Six distinct batches of human plasma were prepared, each with two samples, and sample extraction was carried out following the sample treatment method. Following treatment, a standard solution comprising analytes at two concentrations of LQC and HQC, as well as an internal standard solution, was added to the sample. LQC and HQC samples were analyzed concurrently with an IS standard solution in methanol to calculate the impact of the sample matrix.

Table 4 summarizes the recovery and matrix effects of vancomycin in human plasma.

3.2.7. Stability

The stock solutions were stable within 6 h, 30 days, and 60 days at room temperature and −40 °C. Additionally, the analytes were stable in human plasma following testing in both short- and long-term settings. Table 5 illustrates all the data from the stability investigation.

3.2.8. Carryover and dilution

Carryover was performed by inserting the sample containing the highest concentration (ULOQ) in human plasma into the chromatographic apparatus. After finishing the analysis, immediately inject the blank into the system. Repeat the method six times. To analyze the findings, inject the sample six times at the LLOQ concentration in human plasma.

Dilution validation was performed by creating six simulated plasma samples at a concentration twice that of the HQC concentration, then diluting twice with blank plasma, processing the samples, and analyzing them.

The results indicate that when diluted 2 times, VAL and PHE recovery rates were 89 % and 97 %, respectively (85–115 %) with an RSD value of the recovery rate of 4 % and 6 % (≤ 15 %). Therefore, the developed sample preparation procedure is not affected by dilution. Furthermore, as shown in Fig. 4, there is almost little residual sample impact for VAL, PHE, and IS.

3.3. Application of plasma clinical samples in epileptic patients

This approach was employed for pharmacokinetic evaluations, wherein the quantity of VAL and PHE in the plasma of 105 patients treated with Depakine chrono 500 mg (valproic acid) and Phenytoin 100 mg at Can Tho Central General Hospital following a month of monotherapy or combined therapy was assessed based on the outcomes of all validation parameters.

As shown in Fig. 5, it's evident that the majority of patients who received VAL monotherapy experienced a complete response (86.90 %) with an average blood valproic acid concentration of 59.78 ± 23.90 μg/mL, falling within the therapeutic range of 50–100 μg/mL. However, when patients were concurrently treated with phenytoin, this percentage of VAL decreased to 66.77 %, and the average phenytoin concentration in their blood was 7.64 ± 4.73 μg/mL, which was lower than the required therapeutic range. Notably, none of the patients' VAL and PHE blood levels exceeded the permissible range. Additionally, a definitive correlation between the drug concentration in the bloodstream and the patient's age or weight has not been established.

4. Conclusion

To simultaneously quantify PHE and VAL in human plasma, we developed a UPLC-MS/MS methodology renowned for its remarkable accuracy, robust reproducibility, heightened sensitivity, and specificity. The LLOQ for VAL stands at 3.60 μg/mL, while for PHE, it is 0.72 μg/mL using 200 μL of plasma. This method underwent comprehensive validation in accordance with US-FDA and EMA guidelines. Both matrix effects and extraction recoveries consistently demonstrated stability and reproducibility. Additionally, our study confirmed the stability of these drugs in stock solutions and plasma samples under diverse conditions. Consequently, this method holds promise for pharmacokinetic investigations of VAL and PHE, as well as for routine monitoring in epileptic patients.

Table 3
Precision and accuracy for the analytes in human plasma.

Analyte	Level	QC nominal Conc (μg/mL)	Intra-day (n = 6)			Inter-day (n = 18)		
			Measured Conc (Mean ± SD, μg/mL)	RSD (%)	Accuracy (%)	Measured Conc (Mean ± SD, μg/mL)	RSD (%)	Accuracy (%)
VAL	LLOQ	3.60	3.66 ± 0.20	5.50	101.55	3.88 ± 0.22	5.59	107.73
	LQC	10.80	10.35 ± 0.45	4.34	95.83	11.28 ± 0.25	6.71	104.45
	MQC	60.0	58.17 ± 2.44	4.19	96.95	63.22 ± 0.24	6.40	105.37
	HQC	108.0	115.91 ± 1.66	1.44	107.32	115.46 ± 0.09	2.37	106.91
PHE	LLOQ	0.72	0.73 ± 0.04	5.36	100.77	0.74 ± 0.22	5.86	103.29
	LQC	2.16	2.06 ± 0.13	6.45	95.27	2.18 ± 0.26	7.03	100.94
	MQC	12.0	12.85 ± 0.42	3.28	107.08	12.87 ± 0.12	3.06	107.28
	HQC	21.60	20.00 ± 0.49	2.46	92.60	20.87 ± 0.16	4.46	96.64

Conc: Concentration.

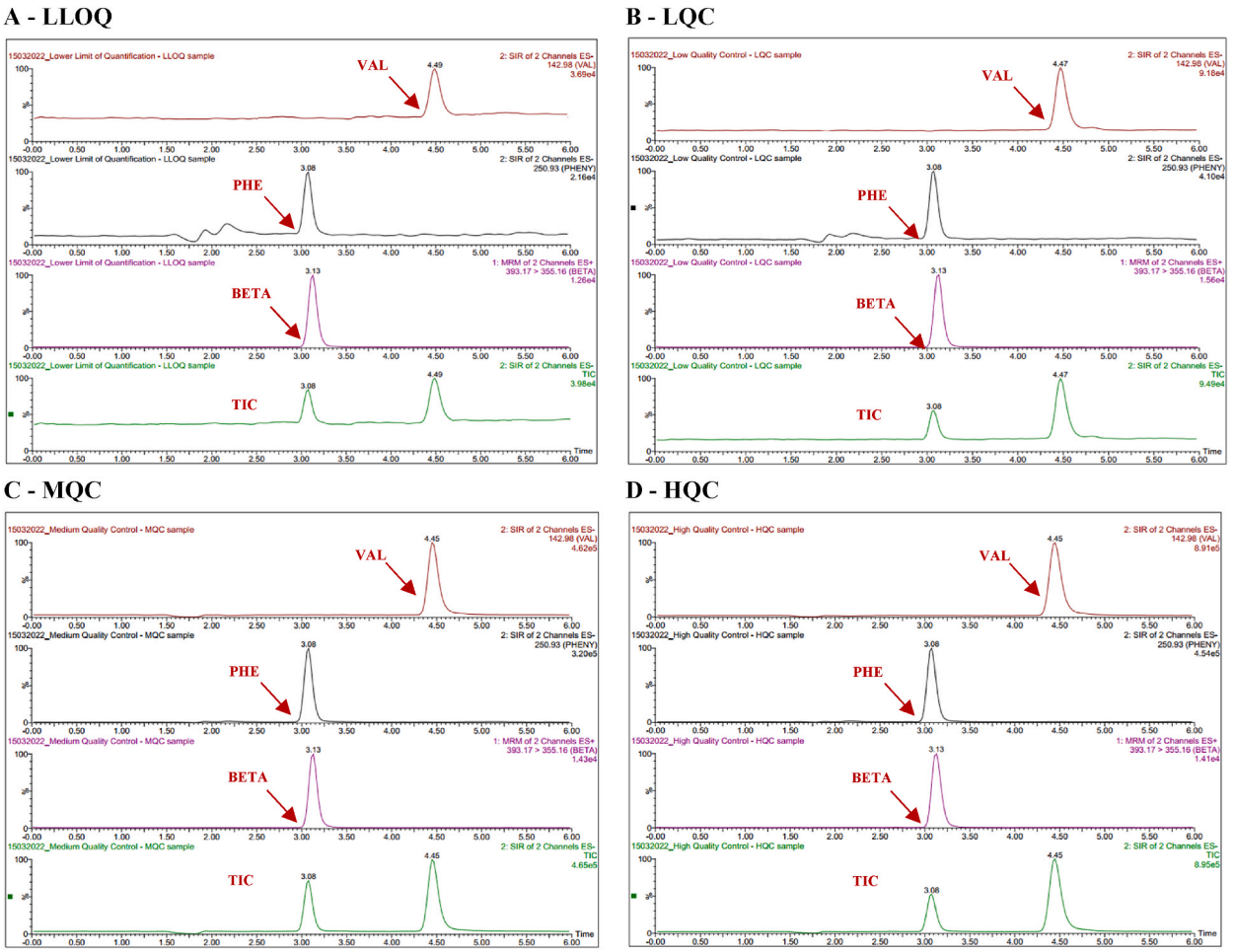

Fig. 3. The chromatograms of these analytes and IS in quality control (QC) samples (A) LLOQ; (B) LQC; (C) MQC; (D) HQC.

Table 4
The extraction recovery and matrix effect of VAL and PHE in human plasma.

Analyte	Level	QC nominal Conc (μg/mL)	Recovery extraction (n = 6)		Matrix effect (n = 6)	
			Mean ± SD (%)	RSD (%)	MF Mean ± SD (%)	RSD (%)
VAL	LQC	10.80	89.00 ± 3.05	3.42	106.76 ± 4.07	3.81
	MQC	60.0	90.87 ± 2.84	3.12	–	–
	HQC	108.0	92.61 ± 3.21	3.47	102.87 ± 5.88	5.72
PHE	LQC	2.16	92.55 ± 4.11	4.44	109.52 ± 5.62	5.13
	MQC	12.0	89.28 ± 3.60	4.03	–	–
	HQC	21.60	90.95 ± 4.24	4.66	96.07 ± 6.29	6.55
IS			86.53 ± 6.55[a]	7.57	99.36 ± 6.64[b]	6.68

[a] n = 18.
[b] n = 12.

Ethics approval

The protocol of this study was approved by the Human Investigation Ethics Committee of the Can Tho University of Medicine and Pharmacy, Vietnam, code 300/PCT-HDDD and 219/PCT- HDDD. The authors confirm that all procedures and experiments in this study were conducted in accordance with the ethical standards outlined in the Helsinki Declaration of 1975, revised in 2013, and complied with national legislation (World Medical Association 2013). Informed consent was obtained from all participants.

Table 5
Stability of analytes and IS in stock standard solution and human plasma.

Storage condition		Analyte	Level	QC nominal Conc (μg/mL)	Mean ± SD (%)	RSD (%)
Stock standard solutions						
Short term	Room temperature for 6h	VAL		500	95.03 ± 3.48	3.66
		PHE		500	93.18 ± 5.96	6.39
		IS		500	98.03 ± 6.62	6.75
Long term	Long-term for 30 days (−40 °C)	VAL		500	93.79 ± 2.67	2.85
		PHE		500	91.65 ± 4.17	4.55
		IS		500	96.81 ± 6.74	6.97
	Long-term for 60 days (−40 °C)	VAL		500	94.24 ± 1.86	1.98
		PHE		500	94.38 ± 1.94	2.06
		IS		500	92.72 ± 3.22	3.47
Analytes in human plasma						
Short term	Room temperature for 6h	VAL	LQC	10.80	104.59 ± 6.51	6.22
			HQC	108.0	93.39 ± 3.75	3.85
		PHE	LQC	2.16	103.00 ± 6.34	6.15
			HQC	21.60	109.21 ± 5.36	4.91
	Autosampler for 24h (10 °C)	VAL	LQC	10.80	110.12 ± 2.62	2.38
			HQC	108.0	105.21 ± 5.24	4.98
		PHE	LQC	2.16	104.17 ± 9.42	9.05
			HQC	21.60	111.21 ± 3.30	2.96
Long term	Three cycles of freezing-defrosting	VAL	LQC	10.80	107.06 ± 2.76	2.58
			HQC	108.0	98.63 ± 2.34	2.37
		PHE	LQC	2.16	110.38 ± 2.30	2.09
			HQC	21.60	107.87 ± 2.58	2.39
	Long-term for 30 days (−40 °C)	VAL	LQC	10.80	92.10 ± 0.97	1.05
			HQC	108.0	95.51 ± 2.34	2.45
		PHE	LQC	2.16	91.02 ± 5.30	5.82
			HQC	21.60	97.27 ± 3.30	3.39
	Long-term for 60 days (−40 °C)	VAL	LQC	10.80	100.81 ± 3.87	3.84
			HQC	108.0	86.58 ± 8.03	9.27
		PHE	LQC	2.16	100.21 ± 7.90	7.89
			HQC	21.60	104.38 ± 6.77	6.49

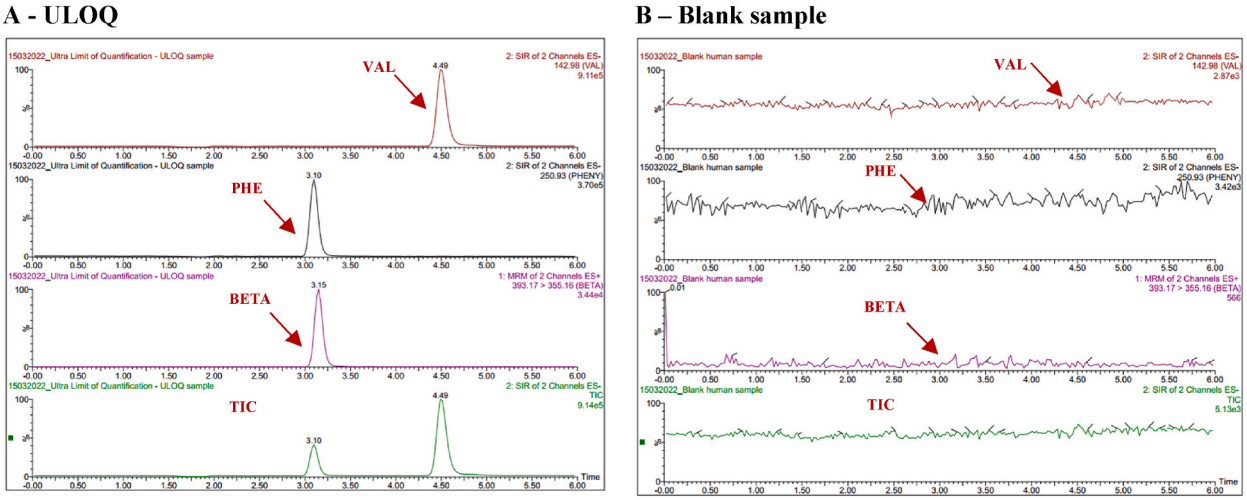

Fig. 4. Chromatograms of the blank sample (B) after Upper Limit of Qualification (ULOQ) injection (A).

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Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

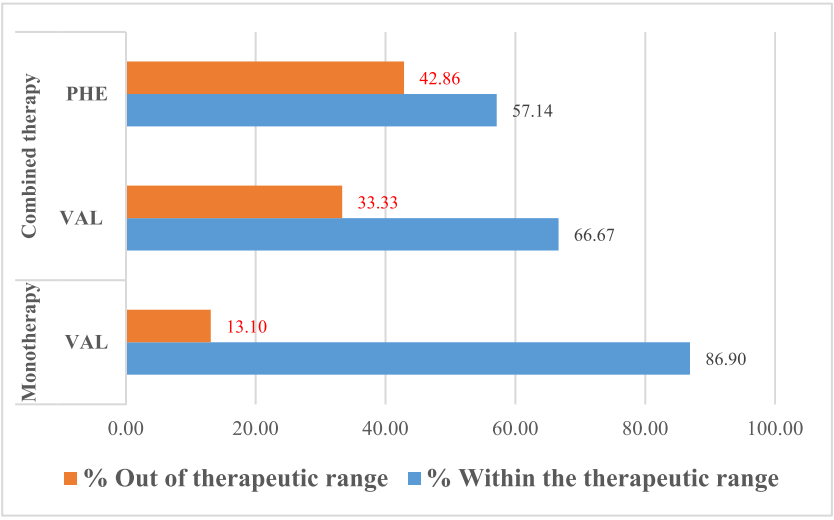

Fig. 5. The results of VAL concentration analysis in 105 individuals with epilepsy following oral Deparkin chrono 500 mg and Phenytoin 100 mg twice day for one month.

CRediT authorship contribution statement

Sil Thanh Nguyen: Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Duy Nguyen Ho:** Validation, Investigation. **Thi Anh Huynh Huynh:** Validation, Investigation. **Huyen Thu Thi Nguyen:** Writing – original draft, Formal analysis. **Ngan Kim Thi Ly:** Visualization, Resources, Methodology. **Minh Van Le:** Visualization, Resources, Methodology. **Tho Vinh Minh Chau Do:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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