

# Development and validation of a new HPLC method for valproic acid determination in human plasma and its application to a therapeutic drug monitoring study

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## Abstract

The aim of this study was to develop a new, simple and reliable high performance liquid chromatography (HPLC) method for analysis of valproic acid (VPA) in human plasma and apply to it to a therapeutic drug monitoring study. Also, the relationship between plasma-VPA concentrations and the amount of VPA used by patients was aimed to be evaluated.

Plasma samples (0.25 mL) were precipitated with the same volume of acetonitrile and after centrifugation, aliquots were applied to a C18 column (250 mm x 4.6 mm). Mobile phase was prepared with phosphate buffer and acetonitrile (47.5:52.5, v/v). The flow-rate was 1.2 mL/min.

Accuracy was between -2.9 and 3.2% and precision was  $\leq 6.6\%$ . Method was specific and sensitive with a detection limit of 2.2  $\mu\text{g/mL}$  and the average recovery was 94.3%. Calibration curve was linear ( $r^2 > 0.9968$ ) from 10 to 150  $\mu\text{g/mL}$ . Plasma-VPA levels of the epileptic patient population ( $n=33$ ) treated with VPA between 0.5 and 1.5 g/day were also determined.

Patient plasma-VPA concentrations ranged from 2.9 to 166.4  $\mu\text{g/g/mL}$  ( $56.3 \pm 38.8$ ). High RSD% (68.8%) was observed in dose-rated plasma-VPA results. Moreover, VPA plasma levels were found to be outside the recommended treatment range in 30.3% of the patients examined. The procedure described was found to be relatively simple, precise, and applicable for routine therapeutic drug monitoring (TDM) especially in neurology clinics or in toxicology reference laboratories.

The high standard deviation (SD) observed in the dose depended plasma-VPA values of the volunteers proved the importance of TDM during the use of this drug. The results showed that for rational drug use, it is important to identify individual polymorphisms in the CYP2C9, CYP2A6 and CYP2B6 subtypes responsible for VPA metabolism, and to rearrange drug doses taking these enzyme activities into account.

**Key words:** valproic acid, plasma, HPLC-UV, therapeutic drug monitoring, validation

## Introduction

Epilepsy is a common and serious neurological disease affecting people of all ages. It is known that 1% of the world population is affected by this disease (Begley et

al., 2020). The potentially life-threatening symptoms can be successfully treated with one or more antiepileptic drugs in most of the patients (Schmidt and Schachter, 2014). VPA, known as sodium valproate (N-dipropylacetic acid) (Nazeri et al., 2014), is simple, eight carbon branched-chain fatty acid (Fig. 1) with unique anticonvulsant properties against several types of epileptic

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seizures. It is derived from the naturally occurring valeric acid. Since it has a successful pharmacodynamic activity, it has been widely prescribed over the world for over 40 years. VPA is also used in the treatment of migraine, bipolar, mood disorders, anxiety and other psychiatric disorders (Chateauvieux et al., 2010). It has also been investigated for its use as an adjuvant agent in cancer, HIV treatment and some neurodegenerative diseases because of its inhibitory effect on histone deacetylase (HDAC) (Terbach and Williams, 2009).

Despite these benefits, the VPA dose requirements are rather variable. There is a 10-fold difference in the average dose in adults and interactions with other drugs are common. Although there are some literature data showing that both, the clinical and toxic effects of this drug are not strongly associated with plasma concentration, drug monitoring during treatment is very important and widely used (Blanco-Serrano et al., 1999). In addition, VPA treatment also has shown some serious life-threatening adverse reactions, including hepatotoxicity, teratogenicity, and pancreatitis. Children are reported to be at greater risk of hepatotoxicity than in adults during VPA treatment (Chateauvieux et al., 2010).

Furthermore, hyperammonemia has proven to be an adverse drug reaction in VPA treatment (Aires et al., 2011).

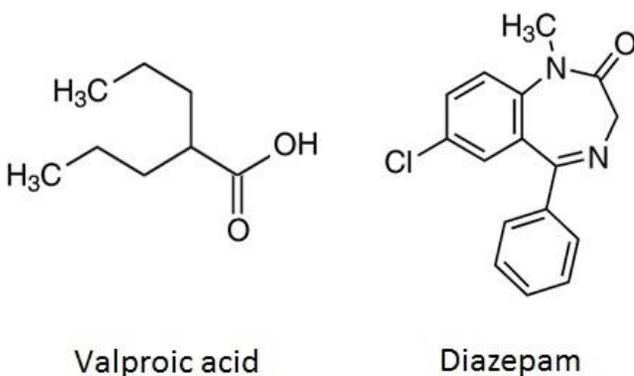


Fig. 1. Chemical structures of VPA and the internal standard DZP.

High % of VPA binding with plasma proteins (87-95%) causes its low clearance (6-20 mL/h/kg) (Leppik, 2006). Therapeutic serum/plasma concentrations range from 20 to 100 µg/mL, and toxic serum/plasma concentrations were between 120 to 150 µg/mL (Regenthal et al., 1999).

High performance liquid chromatography (HPLC) with ultraviolet (UV) detection (Amini et al., 2006; Chen et al., 2012; Kishore et al., 2003; Nazeri et al., 2014), fluorescence detection (Jain et al., 2007; Lin et al., 2004), mass spectrometry (Gao et al., 2011; Jain et al., 2007), gas chromatography flame ionisation detection (Nazeri et al., 2014) and capillary electrophoresis coupled with contactless conductivity detection (Belin et al., 2007; Pham

et al., 2012) are the methods that were used for determination of VPA in human serum/plasma. In addition to that, gas chromatography has been also used due to the volatility property of VPA (Fazeli-Bakhtiyari et al., 2015).

In the literature, there are few published studies for determination of VPA in human blood by HPLC-UV technique. Nazeri et al. (2014) developed new analytical methods based on HPLC-UV and GC-FID and compared the serum-VPA results obtained with these methods. In their study, a C<sub>18</sub> column (150 mm x 4.6 mm I.D.) set at 40 °C was used for separation. The UV detector was set at 210 nm. 20 mM phosphate buffer (pH 3.0) and acetonitrile-water 65:35 (v/v) were used as a mobile phase. Butabarbital (20 µg/mL) was used as an internal standard. The sample preparation procedure was based on treating 200 µL of plasma sample with 200 µL of HCl (1 N). Subsequently, extraction with 500 µL of dichloromethane was done. After, the centrifugation step, the separated organic layer was evaporated under nitrogen at 40 °C. The residue was dissolved in methanol and 20 µL aliquot was applied to HPLC. VPA was detected at 13.6 min. Precision was estimated to be between 3.86 and 6.77%. Recovery was found to be 93.3±3.1%.

Amini et al. (2006) developed a method for VPA determination in plasma with HPLC-UV. In this study, they developed and applied two-stage extraction method, plasma extraction and back extraction, respectively. N-Hexane and diluted triethyl amine in acidic condition were used as extraction solvents. Separation was achieved with an CN (4.6 x 250 mm) column and acetonitrile:40 mM aqueous sodium dihydrogen phosphate (30:70, v/v), pH 3.5 was used as a mobile phase. Analyses were run at a flow-rate of 1 mL/min and UV detection was achieved at 210 nm. Quantification limit was calculated as 1.25 µg/mL. Calibration curves were linear in the range 5-320 µg/mL ( $r^2 > 0.999$ ) and the analysis run time was <11 min.

Another method for VPA determination in human plasma was developed by Kishore et al. (2003). Analytical separation was achieved with a reverse phase C<sub>18</sub> analytic column at 50 °C. Diazepam (DZP) (Fig. 1) was used as an internal standard. 50 mM phosphate buffer (pH 3.0) and acetonitrile (55:45, v/v) were used as a mobile phase. The flow-rate was 1.2 mL/min. The method was linear over a concentration range of 20 to 160 µg/mL for VPA. The UV detector was set at 210 nm for VPA. Precision was <5.4%. These method values were also checked with patient plasma samples containing VPA.

In order to determine VPA and its major metabolite 4-ene-VPA from human plasma, Chen et al. (2012) developed another analysis method and investigated the hepatotoxicity risks of these substances. Octanoic acid was used as internal standard. This method was used for investigation of plasma VPA and 4-ene-VPA levels in a group of Chinese epilepsy patients (n=64). Method was linear in a range between 5 µg/mL and 200 µg/mL for VPA, in plasma. The mobile phase (76:24, v/v) consisting of methanol and water was applied to the C<sub>18</sub> column (150 mm x 4.6 mm ID, 5 mm ps.s) at room

temperature. UV detection was performed at a wavelength of 254 nm and the flow rate was 1 mL/min. For analytes

extraction, plasma samples were acidified with 200  $\mu$ L HCl (1 M) and then treated with 1.5 mL ethyl acetate for 5 min. Afterwards, acidified samples were centrifuged at 4000 g for 10 min. The supernatant of the samples was then transferred to a centrifuge tube containing 50 mL of 2,4'-dibromoacetophenone solution (12 mg/mL in methanol) and 10 mL of triethylamine. The mixture was held in a water bath at 70 °C for 40 min and then it was evaporated under nitrogen gas. Finally, the residue was resuspended with 100  $\mu$ L of mobile phase and loaded into the HPLC for analysis.

Some techniques based on protein precipitation, liquid-liquid extraction (LLE) (Swenberg et al., 1987), solid phase extraction (SPE) (Ahmadkhaniha et al., 2007), solid phase microextraction (SPME) (Deng et al., 2006; Krogh et al., 1995) were used for preparation of biological samples for chromatographic analyses.

The aim of this article was development of a simple and reliable chromatographic method and its validation by performing linearity, repeatability, sensitivity, recovery and robustness tests according to ICH Q2 guideline (ICH 2005). It was also planned to develop a simple and rapid sample preparation method for plasma monitoring of VPA without any derivatization. For plasma-VPA analysis, a broad linear range including therapeutic, sub-therapeutic and overdose VPA concentrations was intended to be included. This method was also intended to be used in a therapeutic drug monitoring study to determine VPA levels in plasma samples of VPA-treated patients. It was also aimed to determine plasma-drug concentrations of VPA-treated epilepsy patients and to analyze the relationship between VPA dose and plasma-VPA levels.

## Material and methods

### *Chemicals and reagents*

The chemical standard of VPA was kindly donated by VEM Pharmaceuticals company (Istanbul, Turkey). Diazepam (DZP) was donated from Ankara University, Institute of Forensic Science. HPLC grade methanol and acetonitrile were obtained from Sigma-Aldrich (MO, USA). Analytical grade potassium dihydrogen phosphate and orthophosphoric acid were purchased from Merck (Darmstadt, Germany). Membrane filter (47 mm, 0.45 pore size) used for filtration of the mobile phase was obtained from Millipore (Massachusetts, USA). Ultrapure water was obtained from the Elga Purelab Water Purification System (Lane End, Buckinghamshire, UK) used throughout the experiment.

### *Selection of internal standard*

Some pharmaceutical chemicals such as: fluphenazine, carbamazepine, opipramol, imipramine, sildenafil and DZP, were tested in order to obtain an internal standard (ISTD) in this study. Carbamazepine, opipramol and imipramine did not demonstrate acceptable UV intensity. Furthermore, fluphenazine and sildenafil did not show appropriate retention times. Finally, it was decided to use DZP as an internal standard because of its high UV sensitivity and its retention time which was compatible with VPA. The high peak sharpness obtained from the chromatograms was another important factor. In addition, the obtained extraction recovery values were shown to be acceptable and reproducible for ISTD. Finally, it was decided to use DZP as an ISTD in the method.

### *Instrumentation and chromatographic conditions*

The separation and quantification were performed by Agilent 1100 series (California, USA) high-performance liquid chromatography (HPLC) equipped with a degasser (G1322A, Degasser), a gradient pump (G1311A, QuadPump), a column oven (G1316A, Colcom), a manual injector (Rheodyne 7725i) with 20  $\mu$ L loop volume, and an UV detector (G1314A, VWD). Agilent Chemstation A.08.03 software was used for the system control and the data integration (Palo Alto, USA). Separation was performed by a reverse phase C<sub>18</sub> analytical column (250 mm x 4.6 mm I.D.) packed with 5  $\mu$ m p.s. ODS (Hichrom, Japan).

Optimum analytical conditions were determined after an optimization procedure was performed for the column, the mobile phase content and the wavelength selection. The UV detector was adjusted to 213 and 230 nm for determination of VPA and DZP, respectively. These values were obtained from the UV region screening test results (Fig. 2).

The mobile phase composed of 20 mM KH<sub>2</sub>PO<sub>4</sub> and 0.1% triethylamine:acetonitrile (47.5:52.5, v/v) was filtered through a 0.45  $\mu$ m membrane filter (Illinois, USA).. Mobile phase pH was adjusted to 4.0 with orthophosphoric acid (1 M). It was degassed in an ultrasonic bath for 30 minutes before each use. The mobile phase flow rate was 1.2 mL/min and it was isocratically applied to the column at 40 °C. Determination of VPA concentrations was carried out considering the ISTD peak ratios, so the unknown concentrations of VPA in these solutions were quantified using linear regression of response (drug/ISTD peak area) versus DZP concentrations.

### *Preparation of stock solutions and working standards*

The main stock of VPA (2 mg/mL) was prepared in methanol and stored at -20 °C. It was seen that the stock solution was chemically stable for at least 1 month.

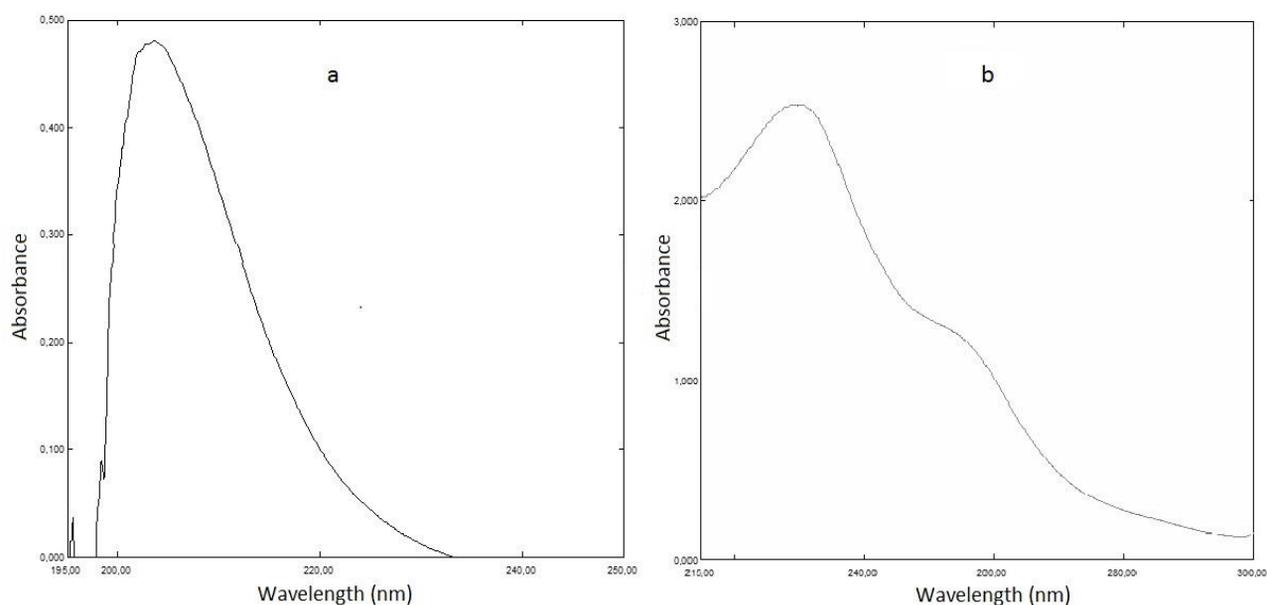


Fig. 2. UV spectrum screening of VPA (a) and DZP (b).

Working standards prepared from VPA stock solution were: 0.25, 0.50, 1.00, 1.50, 2.25, 3.00 and 3.75  $\mu\text{g/mL}$ .

Quality control samples of VPA into drug-free human plasma (10, 20, 40, 60, 90, 120, and 150  $\mu\text{g/mL}$ ) were freshly prepared in order to evaluate the matrix effect. Similarly, for method repeatability studies, VPA-quality control standards in plasma were prepared in concentrations: 20, 60 and 120  $\mu\text{g/mL}$ . The same sample preparation protocol was used for detection limit (LOD), quantification (LOQ), recovery and robustness tests.

The main ISTD stock solution (2 mg/mL) was diluted daily with methanol in order to obtain the working solution (200  $\mu\text{g/mL}$ ).

#### *Preparation of quality control samples and real plasma samples*

Plasma samples were obtained from 33 patients who were under VPA treatment at the Department of Neurology, Medical School, Sivas Cumhuriyet University.

Blood samples (1.5 mL) from epileptic patients with plasma steady-state concentrations of VPA were collected in vacuum tubes containing  $\text{Na}_2\text{EDTA}$ . Samples were taken 12 hours after the last drug intake. After the samples were centrifuged at 4000 rpm for 5 minutes, 250  $\mu\text{L}$  of the supernatant were placed in 1.5 mL eppendorf tubes. The patients' plasma samples and blank samples were stored at  $-20^\circ\text{C}$  until analysed. Analysis of each patient's plasma sample was performed in less than one month.

All clinical experiments were approved by the Clinical Research Investigation Ethics Committee of School of Medicine, Sivas Cumhuriyet University, Sivas, Turkey (The Decision Number: 2018 - 01/21 dated on 10 of January 2018) and these clinical researches with

conducted in accordance with the Declaration of Helsinki and its subsequent revisions. Prior to inclusion in the research, the informed consent of research was obtained from all volunteers who contributed to this study with plasma samples.

As an ISTD, 10  $\mu\text{L}$  DZP (1 mg/mL) and 10  $\mu\text{L}$  VPA (only for making of quality control samples) were added onto 250  $\mu\text{L}$  plasma sample and then it was mixed with 250  $\mu\text{L}$  acetonitrile in order to precipitate the plasma proteins. After the sample tubes were mixed by vortex mixture at 1200 rpm for 2 min, they were centrifuged at 13000 rpm for 3 min. Finally, the upper phase (100  $\mu\text{L}$ ) was manually loaded to the liquid chromatograph.

#### *Statistical analysis*

Statistical analysis was done by using IBM SPSS software version 22. Results were presented as the mean  $\pm$  SD.

## **Results and discussion**

#### *Method validation*

The developed analytical method was validated to demonstrate the specificity and selectivity, linearity, accuracy and precision, limit of detection (LOD) and limit of quantification (LOQ), recovery and robustness. Intraday and inter-day validation protocols were applied considering the reproducibility of the method in order to obtain accurate and precise measurements in agreement with ICH Q2 guideline (ICH 2005).

#### *Specificity and selectivity*

The method showed good chromatographic specificity without endogenous interference at the retention times of

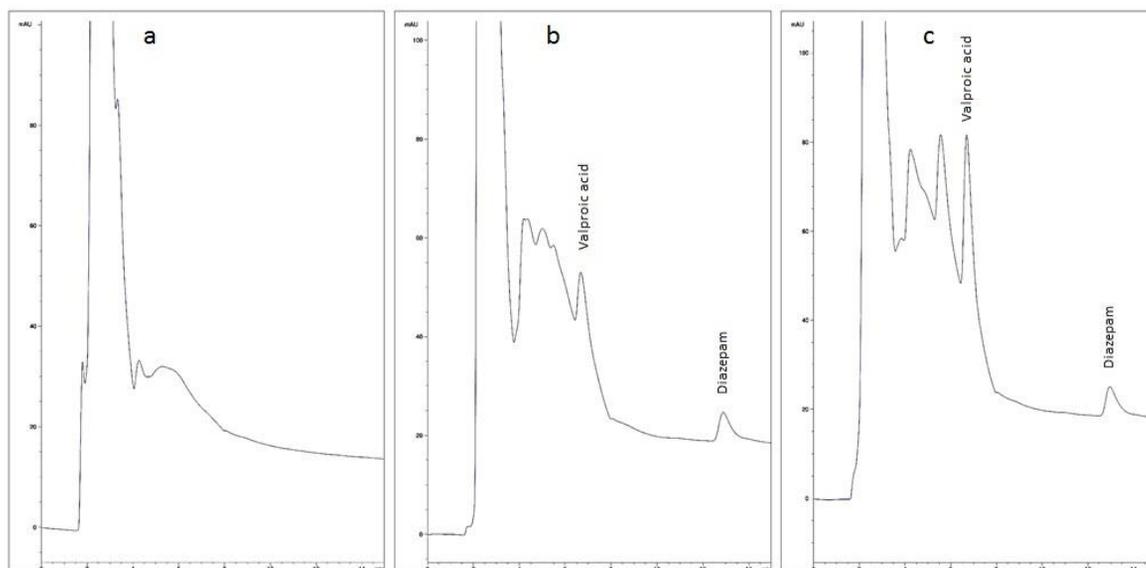


Fig. 3. a) A chromatogram sample of blank plasma used for establishment of the quality control standards in the validation studies. b) A chromatogram sample that contained 60 µg/mL of VPA which was prepared by standard addition method used as a quality control sample. c) A chromatogram sample obtained from a real patient plasma.

VPA and DZP in plasma (6.7 and 13.0 min, respectively). The chromatograms of the blank, spiked and real patient sample are illustrated in Fig. 3a, 3b and 3c, respectively.

#### Linearity

After the chromatographic conditions were determined, the matrix-based calibration curve was plotted in the range of 10 to 150 µg/mL according to the peak area ratios of VPA to ISTD. VPA concentration of 10, 20, 40, 60, 90, 120 and 150 µg/mL

conducted with 3 individual replicates were prepared by standard addition method in plasma and injected to HPLC. These concentrations were chosen with the aim to cover sub-therapeutic, therapeutic and toxic plasma levels of the VPA.

The method showed an excellent correlation coefficient ( $r^2=0.9996$ ). Chromatographic characteristics related with the linearity and system suitability parameters of the method are given in Table 1. Also, the linearity result showed that the method calibration covered sub-therapeutic, therapeutic and toxic plasma concentrations of VPA.

Table 1. Chromatographic characteristics and system suitability parameters of the method.

| Analyte | Wavelength (nm) | T <sub>R</sub> (min) | LOD (µg/mL) | LOQ (µg/mL) | Linear Range (µg/mL) | Calibration equation | r <sup>2</sup> | k'  | N     |
|---------|-----------------|----------------------|-------------|-------------|----------------------|----------------------|----------------|-----|-------|
| VPA     | 213             | 6.7                  | 2.2         | 6.6         | 10 - 150             | y = 0.0049x + 0.050  | 0.9968         | 2.8 | 8472  |
| DZP     | 230             | 13.0                 | -           | -           | -                    |                      |                | 4.2 | 12879 |

Abbreviations: k': Capacity factor; T<sub>R</sub>: Retention time; r<sup>2</sup>: Correlation coefficient; N: Theoretical plate number.

Table 2. Confidence parameters including intraday and inter-day precision and accuracy values.

| Conc.<br>(µg/mL) | Intraday    |  |                     |                   | Inter-day   |  |                     |                   | Average<br>Recovery<br>(%) |
|------------------|-------------|--|---------------------|-------------------|-------------|--|---------------------|-------------------|----------------------------|
|                  | No.<br>Obs. | Estimated<br>conc. $\bar{x} \pm SD$<br>(µg/mL) | Precision<br>(RSD%) | Accuracy<br>(RE%) | No.<br>Obs. | Estimated<br>conc. $\bar{x} \pm SD$<br>(µg/mL) | Precision<br>(RSD%) | Accuracy<br>(RE%) |                            |
| 20               | 5           | 19.9±0.9                                       | 4.5                 | -0.4              | 5           | 19.4±1.3                                       | 6.6                 | -2.9              | 107.0                      |
| 60               | 5           | 61.9±2.4                                       | 3.9                 | 3.2               | 5           | 59.0±2.8                                       | 4.8                 | -1.6              | 94.5                       |
| 120              | 5           | 119.9±3.4                                      | 2.8                 | -0.1              | 5           | 122.7±4.0                                      | 3.3                 | 2.3               | 81.4                       |

Abbreviations: Conc.: Concentration; No. Obs. number of observation.

#### Accuracy and precision

The accuracy, defined as the relative error (RE%) was calculated as the percentage difference between the added and found VPA quantity by five separate replicates, both, intraday and inter-day. The precision, defined as relative standard deviation (RSD%), was calculated by individual five separate replicates of VPA, both, intraday and inter-day. Five replicate spiked individual samples were also prepared and analysed intraday and inter-day at the three different concentration levels (20, 60 and 120 µg/mL).

The data obtained from the accuracy and precision tests, performed intraday and inter-day, with quality control samples established in the blank plasma samples by standard addition method, showed low RSD% values ( $\leq 6.6\%$  and  $\leq 4.5\%$  for interday and intraday, respectively). Also, the obtained RE% values that were between -2.9 and 2.3% for interday, and -0.4 and 3.2% for intraday test values showed significant results in terms of accuracy.

#### Sensitivity

LOD and LOQ were calculated according to the ICH Q2 recommendations based on SD of the response and the slope of the calibration graph (ICH 2005). Calculation equations for LOD and LOQ are given below:

$$\text{LOD} = 3.3\sigma/S \quad (\text{Eq. 1})$$

$$\text{LOQ} = 10\sigma/S \quad (\text{Eq. 2})$$

( $\sigma$ : The SD of the response; S: The slope of the calibration curve).

Concentration of 5 µg/mL VPA was used for the

sensitivity test. In this step, 10 quality control (QC) samples were prepared separately and analysed in series. The results of LOD and LOQ values were obtained by measuring all 10 individual quality control (QC) samples.

The obtained LOD and LOQ values were 2.2 µg/mL and 6.6 µg/mL, respectively. It was seen that these values were under the lowest calibration point which was 10 µg/mL. LOD and LOQ values proved that the method has enough capacity for sensitive measurement of VPA in plasma. Sensitivity parameters are also given in Table 1.

#### Recovery

This test was conducted by comparison of the pre-extraction spikes of VPA and ISTD values to the post-extraction. Five individual replicates of spiked samples in the low, middle and high concentrations of VPA and ISTD in plasma (20, 60 and, 120 µg/mL) were prepared and then analysed, according to the described method. Another five individual replicates at the low, middle and high concentrations of VPA and ISTD in plasma (20, 60 and, 120 µg/mL, respectively) were also prepared with post-extraction method and analysed. Extraction procedure was carried out as described before in sample preparation step.

Recovery test results were between 81.4 and 107.0% (94.3±12.80%) (Table 2). Obtained results in the extraction procedure demonstrated good efficiency. Although the extraction procedure of the method was not complicated and there was no need for sophisticated instruments, it had high extraction ability and yielded significant results. Some of the advantages of this method are: simplicity, fast performance, reproducibility and applicability to many laboratories with different conditions.

#### Robustness

The response of the method to the change of UV wavelength ( $\pm 3$  nm), mobile phase flow rate ( $\pm 0.1$  mL/min), mobile phase solvent content ( $\pm 5\%$ ) and column temperature ( $\pm 4$  °C) were investigated.

No significant changes in the analytical signals were observed upon changing UV wavelength value ( $\pm 3$  nm), mobile phase flow-rate ( $\pm 0.1$  mL/min), mobile phase organic solvent ingredient ( $\pm 5\%$ ), and column temperature ( $\pm 5$  °C). Additionally, change of analysts, columns and source of chemicals did not lead to significant changes in chromatographic signals. The robustness test results showed that the method had a high ability to produce acceptable accuracy and accuracy data.

### Stability

The stability of QC plasma samples (20, 60 and 120  $\mu\text{g/mL}$ ) and analytes in stock solutions under several conditions were assessed. Stability of the stock solutions at room temperature was evaluated after 1, 2, 3 and 4 weeks periods. The freeze-thaw stability test was performed with three QC sample concentrations (20, 60 and 120  $\mu\text{g/g/mL}$ ) after running 5 repeated freeze-thaw periods. The long-term stability test was carried out for 1, 2 and 3 months using QC samples maintained at  $-20$  °C. Neither significant decrease nor degradation were observed in the concentration of VPA when stored under before mentioned different conditions. The relative SD in all samples was less than 3.8%.

As stated in Introduction part, there are some studies about determination of VPA in human plasma by HPLC (Amini et al., 2006; Chen et al., 2012; Kishore et al., 2003; Nazeri et al., 2014). It is seen that these methods have different optimization values and validation results. In addition, different techniques and methods were used for the sample preparation. Although, our developed method included only one extraction step, it had very high recovery value (94.3%). This value gives it greater chance to be used in real studies and also in routine analyses. In our study, the results of the tests performed before plasma sample analysis were used for VPA and DZP due to the high absorbance values obtained at 213 nm and 230 nm wavelengths. The precision of 6.6% showed that the method used was in accordance with other methods from literature data. In addition, the obtained accuracy values which were between  $-2.9$  and  $3.2\%$ , proved the reproducibility of the method. The LOD value of  $2.2$   $\mu\text{g/mL}$  clearly demonstrated the sensitivity of the method. It was below the lowest calibration point which was  $10$   $\mu\text{g/mL}$ . The method linearity in the range from  $10$  to  $150$   $\mu\text{g/mL}$  also enabled the method to be used safely at different dose levels.

### Measurement of VPA plasma levels in volunteer samples

Drug levels in plasma samples, taken from volunteers (patients) who were receiving between 250 and 1500 mg/day VPA orally for epilepsy treatment, were monitored by our developed and validated HPLC method. Assays were performed with 0.25 mL plasma samples from volunteers with steady-state concentration of VPA. No problems were encountered during the monitoring of the analytes in these samples. Interference with any other analyte was not observed during the separation of the analytes.

The observed VPA and DZP peaks were very sharp and separate. Although plasma samples were subjected to a simple protein precipitation process, no endogenous peaks interfering with the analytes monitored were observed. Daily amounts of VPA used, VPA plasma levels and dose-proportional plasma ratios are given in Table 3 and Fig. 4. Dose-proportional VPA plasma results have a meaning in that they show the activity of the enzymes (CYP2C9, CYP2A6 and CYP2B6 subtypes) responsible for the metabolism of VPA. Descriptive statistical analysis results for the data obtained from volunteer plasma are given in Table 4.

A sample chromatogram given in Fig. 3c represents the real patient sample separation. All plasma samples were successfully analysed and their VPA levels were also measured. There were 33 voluntary patients' plasma samples, who have been treated with VPA between 250 and 1500 mg/day, orally, while the average dose/day of VPA was 900 mg. The detected average plasma VPA level was  $56.3$   $\mu\text{g/mL}$  and SD of plasma results was  $38.8$  (68.8, RSD%). SD and RSD% values were higher than expected due to the deviation between plasma VPA results.

Since the therapeutic range of the drug has narrow and has toxicological risks, the distribution of plasma values is of great importance. The high %RSD results obtained from plasma-VPA values proportional to the VPA administration dose indicated the importance of TDM administration for this drug. The unexpected high levels SD and also RSD% emerged in the result. The dose-proportional results of patient plasma VPA levels were  $67.0 \pm 37.6$   $\mu\text{g/mL}$  (56.1% RSD). The metabolic differences of enzymes responsible for the biotransformation of this drug between individuals are thought to play an important role in the emergence of this difference. In addition, the bioavailability of this drug might be negatively affected by the first pass effect. This situation leads to reduction in the pharmacological and pharmacodynamic effect expected from the VPA treatment. Plasma VPA results were evaluated statistically by considering the daily doses of this medicine used by patients. The average female and male plasma VPA results were  $71.2$  and  $60.0$   $\mu\text{g/mL/g}$ , respectively. Although the results demonstrated that men show higher metabolic activity compared to women, significant difference was found between male and female

patients' VPA plasma results ( $p>0.05$ ). These findings indicate that the expression and activity levels of liver biotransformation enzymes are not statistically different

in male and female. This surprising result, which is very important from a toxicological point of view, has shown that we need to do further research in order to

Table 3. Samples included in the analysis, daily VPA doses and plasma concentrations.

| N.P. | Dose (g) | Plasma-VPA conc. ( $\mu\text{g/mL}$ ) | Plasma-VPA conc. ( $\mu\text{g/mL}$ )/Dose (g) | N.P. | Dose (g) | Plasma-VPA conc. ( $\mu\text{g/mL}$ ) | Plasma-VPA conc. ( $\mu\text{g/mL}$ )/Dose (g) | N.P. | Dose (g) | Plasma-VPA conc. ( $\mu\text{g/mL}$ ) | Plasma-VPA conc. ( $\mu\text{g/mL}$ )/Dose (g) |
|------|----------|---------------------------------------|--|------|----------|---------------------------------------|--|------|----------|---------------------------------------|--|
| 1    | 0.5      | 48.3                                  | 96.6   | 12   | 0.6      | 53.1                                  | 88.5   | 23   | 1.5      | 9.4                                   | 6.3  |
| 2    | 1.0      | 76.4                                  | 76.4   | 13   | 1.0      | 62.3                                  | 62.3   | 24   | 1.5      | 149.7                                 | 99.8   |
| 3    | 1.0      | 53.6                                  | 53.6   | 14   | 0.25     | 29.9                                  | 119.7  | 25   | 1.5      | 6.6                                   | 4.4  |
| 4    | 1.0      | 18.0                                  | 18.0   | 15   | 1.0      | 34.9                                  | 34.9   | 26   | 1.0      | 70.5                                  | 70.5   |
| 5    | 0.45     | 14.6                                  | 32.4   | 16   | 1.0      | 55.7                                  | 55.7   | 27   | 1.0      | 81.4                                  | 81.4   |
| 6    | 0.6      | 32.5                                  | 54.2   | 17   | 0.8      | 64.9                                  | 81.1   | 28   | 0.9      | 94.1                                  | 104.5  |
| 7    | 1.0      | 66.0                                  | 66.0   | 18   | 0.6      | 53.3                                  | 88.9   | 29   | 1.0      | 86.5                                  | 86.5   |
| 8    | 1.5      | 7.2                                   | 4.8  | 19   | 0.5      | 51.0                                  | 101.9  | 30   | 1.0      | 72.9                                  | 72.9   |
| 9    | 0.5      | 54.3                                  | 108.6  | 20   | 0.4      | 2.9                                   | 7.1  | 31   | 1.5      | 166.3                                 | 110.9  |
| 10   | 1.0      | 10.3                                  | 10.3   | 21   | 1.0      | 41.5                                  | 41.5   | 32   | 0.75     | 98.3                                  | 131.0  |
| 11   | 0.6      | 58.2                                  | 96.9   | 22   | 0.75     | 22.8                                  | 30.4   | 33   | 1.0      | 111.2                                 | 111.2  |

N.P.: Number of patient; conc.: concentration.

Table 4. Comparative representation of the descriptive statistics of VPA patients' plasma results obtained by two different methods.

|      | Analysis metod-A                       |   | Analysis method-B                      |   |
|------|--|---|--|---|
|      | Plasma-VPA result ( $\mu\text{g/mL}$ ) | Plasma-VPA result ( $\mu\text{g/mL}$ )/Dose (g) | Plasma-VPA result ( $\mu\text{g/mL}$ ) | Plasma-VPA result ( $\mu\text{g/mL}$ )/Dose (g) |
| Mean | 56.3                                   | 67.0  | 51.8                                   | 61.4  |
| SD   | 38.8                                   | 37.6  | 37.4                                   | 36.4  |
| RSD% | 68.8                                   | 56.1  | 72.3                                   | 59.4  |

Abbreviations: Analysis metod-A: Our developed and validated analysis method; Analysis method-B: Analysis method routinely used by the hospital for plasma-VPA analysis

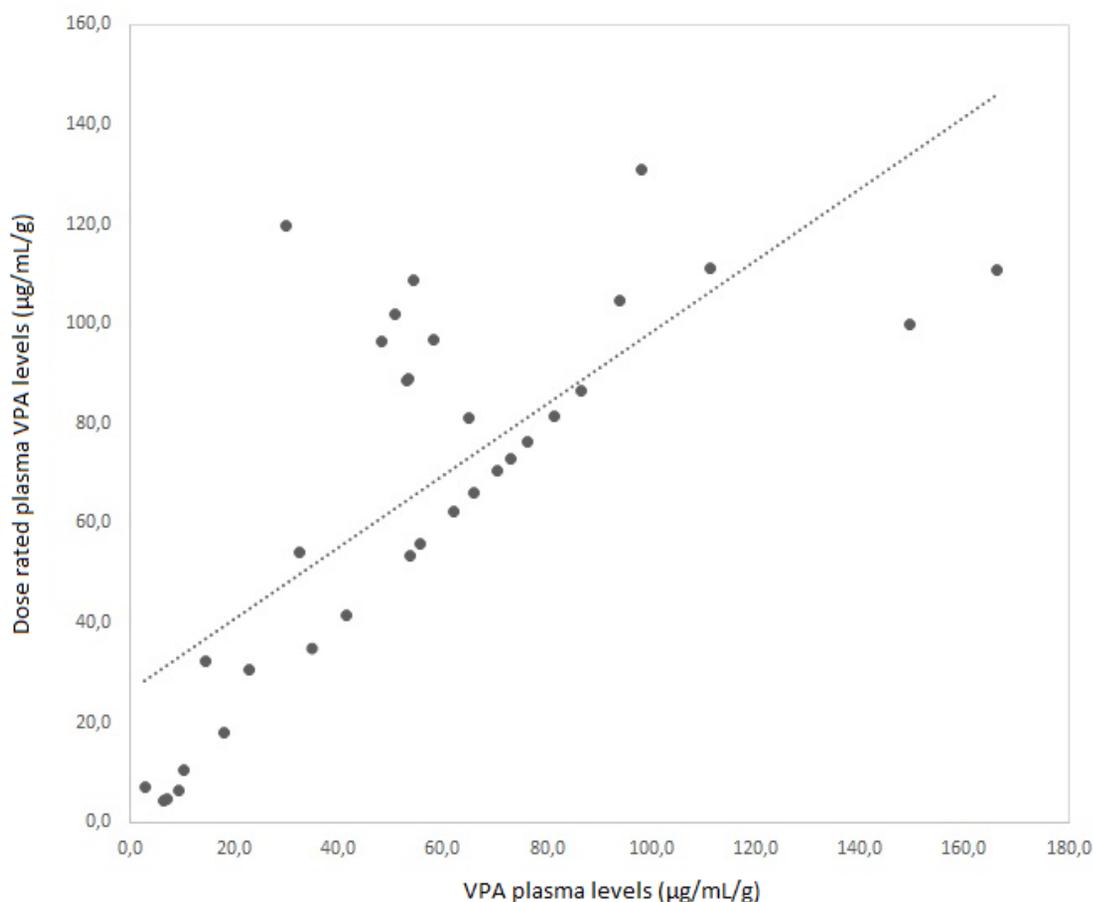


Fig. 4. Comparison of the VPA plasma levels results and the VPA dose rated plasma levels results.

demonstrate the differences in activity of metabolic enzymes (CYP2C9, CYP2A6 and CYP2B6 subtypes). Although many studies have reported that women have higher clearance activity than men, in our results, it was observed that VPA plasma values of women were higher than men (Kobayashi et al., 2017).

Recommended plasma values for the treatment with VPA are between 20 and 100 µg/mL. The study showed that 10 out of 33 (30.3%) patients treated with VPA had plasma concentrations below these values. This result in our study showed that 20.2% of the patient population followed up did not reach an effective blood concentration and continued their treatment. However, this is very important because the patients show that they continue to undergo treatment for VPA at a lower level than they need. This means that, despite drug intake, serious symptoms of the disease cannot be prevented. Also, it shows that 3 patients were also treated with high-dose VPA. This situation is very important in terms of public health because the treatment has serious toxicological risks especially hepatotoxicity. It was thought that the effect of biotransformation enzymes on VPA was an important factor in the emergence of this unexpected result. On the other hand, multidrug therapy is thought to be another important cause of the outcome.

In conclusion, this result showed the importance and necessity of TDM during VPA treatment.

VPA levels in plasma samples of 33 patients included in our study were analysed by another analysis method which is immunoassay based. This control analysis is a routine periodic application and is performed at the initial stage of drug treatment or at the dose increase or decrease stages of the drug. This follow-up may also be needed during combined drug therapy or because of complaints related to epilepsy.

This plasma-VPA analysis results officially performed by Faculty of Medicine, Sivas Cumhuriyet University were compared statistically with the results obtained by our suggested method. The obtained descriptive statistical results were given at Table 3. No significant difference was found between the results of plasma VPA levels obtained from both methods ( $p > 0.05$ ). Plasma VPA results from the two methods were rearranged according to the drug doses administered to the patients, and no significant difference was observed in the results from the two methods ( $p > 0.05$ ).

## Conclusion

Our proposed method showed remarkable repeatability test results between -2.9 and 3.2% RE (accuracy) and  $RSD\% \leq 6.6$  (precision), respectively (ICH 2005).

Therefore, the repeatability of the method was very high. This result increases the reliability of the blood results obtained. The sample extraction had a simple and fast single step which provided reliable and excellent recovery values between 81.4 to 107.7%. The simplicity of sample preparation, the appropriate analysis time (<14 minutes) and the high sensitivity of the presented technique made the method very attractive. In our study, almost all of the patients undergoing blood monitoring were using at least one more drug in addition to VPA and were generally receiving multi-drug therapy. Although diazepam is used to treat anxiety, alcohol withdrawal, and seizures, combined treatment of VPA and DZP is rare in the clinical practice because valproic acid is especially used to treat tonic and clonic seizures in epilepsy. Therefore, it was not inconvenient to use DZP as an internal standard in the method. On the other hand, this did not cause any problems in the chromatographic separation and quantitation in any plasma sample. Consequently, this method is rapid, sensitive and reliable.

We strongly recommended this validated method to be used in the routine therapeutic drug analysis of VPA and also it can be adapted for monitoring of overdose/poisoning with this drug. Also, it can be used in suicide cases. The proposed method can be easily applied in routine TDM studies of VPA, also it can be preferred in the bioequivalence, pharmacokinetics and pharmacovigilance studies.

In our study, it was observed that plasma-VPA levels did not show any change related to gender. However, it was also seen that the levels of plasma-VPA which were recorrected according to daily drug doses were highly variable, with high SD and RSD% values: 37.6 and 56.1, respectively. Because of the significant toxicological differences observed between VPA plasma levels and dose-proportional VPA plasma levels, it is planned to investigate the polymorphism rates of CYP2C9, CYP2A6 and CYP2B6 subtypes responsible for VPA metabolism in patients' blood.

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## References

- Ahmadkhanhiha, R., Rastkari, N., Kobarfard, F., Pakdaman, H., Ahmadkhanhiha, O., Kebriaeezadeh, A., 2007. An improved GC method for rapid analysis of valproic acid in human plasma without derivatization. *Iran. J. Pharm. Sci.* 3, 37–42.
- Aires, C.C.P., van Cruchten, A., Ijst, L., de Almeida, I.T., Duran, M., Wanders, R.J.A., Silva, M.F.B., 2011. New insights on the mechanisms of valproate-induced hyperammonemia: inhibition of hepatic N-acetylglutamate synthase activity by valproyl-CoA. *J. Hepatol.* 55, 426–434. Available at: <https://doi.org/10.1016/j.jhep.2010.11.031>.
- Amini, H., Javan, M., Ahmadiani, A., 2006. Development and validation of a sensitive assay of valproic acid in human plasma by high-performance liquid chromatography without prior derivatization. *J. Chromatogr. B. Anal. Technol. Biomed. Life Sci.* 830, 368–371. Available at: <https://doi.org/10.1016/j.jchromb.2005.11.028>.
- Begley, C.E., Famulari, M., Annegers, J.F., Lairson, D.R., Reynolds, T.F., Coan, S., Dubinsky, S., Newmark, M.E., Leibson, C., So, E.L., Rocca, W.A., 2000. The cost of epilepsy in the United States: an estimate from population-based clinical and survey data. *Epilepsia* 41, 342–351. Available at: <https://doi.org/10.1111/j.1528-1157.2000.tb00166.x>.
- Belin, G.K., Krahenbuhl, S., Hauser, P.C., 2007. Direct determination of valproic acid in biological fluids by capillary electrophoresis with contactless conductivity detection. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* 847, 205–209. Available at: <https://doi.org/10.1016/j.jchromb.2006.10.003>.
- Blanco-Serrano, B., Otero, M.J., Santos-Buelga, D., Garcia-Sanchez, M.J., Serrano, J., Dominguez-Gil, A., 1999. Population estimation of valproic acid clearance in adult patients using routine clinical pharmacokinetic data. *Biopharm. Drug Dispos.* 20, 233–240.
- Chateauvieux, S., Morceau, F., Dicato, M., Diederich, M., 2010. Molecular and therapeutic potential and toxicity of valproic acid. *J. Biomed. Biotechnol.* 2010, 479364. Available at: <https://doi.org/10.1155/2010/479364>.
- Chen, Z., Wang, X., Wang, H., Chen, S., Zhou, L., Li, J., Shu, W., Zhou, J., Fang, Z., Zhang, Y., Huang, M., 2012. Simultaneous determination of valproic acid and 2-propyl-4-pentenoic acid for the prediction of clinical adverse effects in Chinese patients with epilepsy. *Seizure* 21, 110–117. Available at: <https://doi.org/https://doi.org/10.1016/j.seizure.2011.10.002>.
- Deng, C., Li, N., Ji, J., Yang, B., Duan, G., Zhang, X., 2006. 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.* 20, 1281–1287. Available at: <https://doi.org/10.1002/rcm.2451>.
- Fazeli-Bakhtiyari, R., Panahi-Azar, V., Sorouraddin, M.H., Jouyban, A., 2015. Determination of valproic acid in human plasma using dispersive liquid-liquid microextraction followed by gas chromatography-flame ionization detection. *Iran. J. Basic Med. Sci.* 18, 979–988.

- Gao, S., Miao, H., Tao, X., Jiang, B., Xiao, Y., Cai, F., Yun, Y., Li, J., Chen, W., 2011. LC-MS/MS method for simultaneous determination of valproic acid and major metabolites in human plasma. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* 879, 1939–1944. Available at: <https://doi.org/10.1016/j.jchromb.2011.05.022>.
- International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, ICH Harmonised Tripartite Guideline, Validation of Analytical Procedures: Text and Methodology Q2(R1), Current Step 4 version, November, n.d. Available at: [https://database.ich.org/sites/default/files/Q2\\_R1\\_Guideline.pdf](https://database.ich.org/sites/default/files/Q2_R1_Guideline.pdf). Last accessed: June, 2019.
- Jain, D.S., Subbaiah, G., Sanyal, M., Shrivastav, P., 2007. A high throughput and selective method for the estimation of valproic acid an antiepileptic drug in human plasma by tandem LC-MS/MS. *Talanta* 72, 80–88. Available at: <https://doi.org/10.1016/j.talanta.2006.09.033>.
- Kishore, P., Rajani Kumar, V., Satyanarayana, V., Krishna, D.R., 2003. HPLC determination of valproic acid in human serum. *Pharmazie* 58, 378–380.
- Kobayashi, K., Abe, C., Endo, M., Kazuki, Y., Oshimura, M., Chiba, K., 2017. Gender difference of hepatic and intestinal CYP3A4 in CYP3A humanized mice generated by a human chromosome-engineering technique. *Drug Metab. Lett.* 11 (1), 60–67. Available at: <https://doi.org/10.2174/1872312811666170404153804>.
- Krogh, M., Johansen, K., Tonnesen, F., Rasmussen, K.E., 1995. Solid-phase microextraction for the determination of the free concentration of valproic acid in human plasma by capillary gas chromatography. *J. Chromatogr. B. Biomed. Appl.* 673, 299–305.
- Leppik, I.E., 2006. Epilepsy in the elderly. *Epilepsia* 47(Suppl 1), 65–70. Available at: <https://doi.org/10.1111/j.1528-1167.2006.00664.x>.
- Lin, M.C., Kou, H.S., Chen, C.-C., Wu, S.M., Wu, H.L., 2004. Simple and sensitive fluorimetric liquid chromatography method for the determination of valproic acid in plasma. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* 810, 169–172. Available at: <https://doi.org/10.1016/j.jchromb.2004.07.025>.
- Nazeri, A., Jalali, M., Aliasgharpour, M., Khosravie, F., 2014. Comparison of Serum Valproic Acid Determination Through Gas and High Performance Liquid Chromatography Methods. *Heal. Scope* 3, e12085. Available at: <https://doi.org/10.17795/jhealthscope-12085>.
- Pham, T.T.T., See, H.H., Morand, R., Krähenbühl, S., Hauser, P.C., 2012. Determination of free and total valproic acid in human plasma by capillary electrophoresis with contactless conductivity detection. *J. Chromatogr. B* 907, 74–78. Available at: <https://doi.org/10.1016/j.jchromb.2012.08.037>.
- Regenthal, R., Krueger, M., Koepfel, C., Preiss, R., 1999. Drug levels: therapeutic and toxic serum/plasma concentrations of common drugs. *J. Clin. Monit. Comput.* 15, 529–544.
- Schmidt, D., Schachter, S.C., 2014. Drug treatment of epilepsy in adults. *BMJ* 348, g254. Available at: <https://doi.org/10.1136/bmj.g254>.
- Swenberg, J.A., Richardson, F.C., Boucheron, J.A., Deal, F.H., Belinsky, S.A., Charbonneau, M., Short, B.G., 1987. High- to low-dose extrapolation: critical determinants involved in the dose response of carcinogenic substances. *Environ. Health Perspect.* 76, 57–63. Available at: <https://doi.org/10.1289/ehp.877657>.
- Terbach, N., Williams, R.S.B., 2009. Structure–function studies for the panacea, valproic acid. *Biochem. Soc. Trans.* 37, 1126–1132. Available at: <https://doi.org/10.1042/BST0371126>.

## Резиме

## Развој и валидација на нов HPLC метод за одредување на валпроична киселина во хумана плазма и негова примена во студија за терапевтски мониторинг на лековите

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**Клучни зборови:** валпроична киселина, плазма, HPLC-UV, терапевтски мониторинг на лекови, валидација

Целта на оваа студија беше да се развие нов, едноставен и сигурен HPLC метод за анализа на валпроична киселина (VPA) во хумана плазма со цел истиот да се примени во рутинскиот терапевтски мониторинг на лековите. Исто така, планирано е да се утврди односот помеѓу плазма концентрацијата на VPA и количината на VPA администрирана кај пациентите.

Плазма примероците (0,25 mL) беа преципитирани со ист волумен на ацетонитрил и по нивно центрифугирање, истите беа аплицирани на C18 колона (250 mm x 4,6 mm). Мобилната фаза содржеше фосфатен пуфер и ацетонитрил (47,5:52,5, v/v) протоколот беше 1,2 mL/min.

Точноста на методот беше помеѓу -2,9 и 3,2%, а прецизноста  $\leq 6,6\%$ . Истиот се карактеризираше со специфичност и чувствителност, со лимит на детекција од 2,2  $\mu\text{g/mL}$ , а просечниот recovery беше 94,3%. Калибрационата крива беше линеарна ( $r^2 > 0,9968$ ) во опсег од 10 до 150  $\mu\text{g/mL}$ . Исто така, беа утврдени и плазма нивоата на VPA на популацијата од пациенти со епилепсија (n=33) третирани со дози на VPA помеѓу 0,5 и 1,5 g/ден.

Концентрациите на VPA во плазмата на пациентите се движеа од 2,9 до 166,4  $\mu\text{g/g/ml}$  (56,3 $\pm$ 38,8). Покрај тоа, плазма нивоата на VPA беа надвор од препорачаниот опсег кај 30,3% од испитаниците. Опишаната постапка е релативно едноставна, прецизна и применлива за рутински терапевтски мониторинг на лекови (TDM), особено во клиниките за неврологија или во референтните лаборатории за токсикологија.

Високата стандардна девијација забележана кај вредностите за плазма концентрациите при прилагодените дози на VPA ја покажува важноста на TDM за време на безбедната употреба на овој лек. Резултатите покажаа дека за рационална употреба на овој лек, од особена важност е да се идентификуваат индивидуалните полиморфизми во подтиповите: CYP2C9, CYP2A6 и CYP2B6 одговорни за метаболизмот на VPA и да се прилагодат дозите на лекот земајќи ја во предвид активноста на овие ензими.