A rapid and simple procedure for monitoring valproic acid by gas chromatography

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Abstract

Valproic acid (VPA), a widely used antiepileptic drug, has a narrow therapeutic range of 50-100 µg/mL and shows large individual variability. It is very important to monitor the trough VPA concentration using a reliable method. The aim of this study was to develop and validate a rapid gas chromatographic (GC) technique for VPA quantification in human plasma and to compare it with the traditional immunoassay method. VPA extraction from human serum was efficient by dichloromethane and hydrochloric acid using octanoic acid as an internal standard. GC analysis was performed using a gas-chromatograph equipped with a flame ionization detector (GC/FID). VPA detection and quantification were accomplished isothermally at 135°C on a Gs-BP 100% dimethylpolysiloxane capillary column (10 m×0.53 mm ID, 2.65 µm film thickness, Supelco, Bellefonte, PA). Injection port and detector temperature were 280°C. Retention times of VPA and internal standard were 1.83 min and 2.33 min, respectively. The calibration curve was linear over the concentration range of 5-320 µg/mL, with a lower limit of detection of 1.25 µg/mL. The internal and inter-day precision was less than 5.3% and 6.1%, respectively, and the accuracy was below 2.8%. VPA recovery was 94.6%. A quick and accurate method for VPA determination in human plasma was developed and validated. It resulted sufficiently selective and sensitive.

Introduction

Valproic acid (2-propylpentanoic acid, VPA) (Figure 1) is a simple eight carbon branched-chain fatty acid with unique anticonvulsant properties and is used in the treatment of epilepsy, bipolar disorder and prophylaxis of migraine headaches.[1-5] VPA acts on dopamine and glutamine neurotransmission as well as an intracellular signaling.[6]

Common adverse effects of valproate treatment include weight gain, gastrointestinal symptoms, sedation, tremor, heart burn, impaired vision, hearing loss, respiratory depression, headache, joint pain and mild elevation of liver enzymes. Severe hepatotoxicity is rare in adults and many adverse effects are dose related and resolve with dose reduction.[7] Overdose in children is usually of accidental origin, whereas in adults it is more likely to be an intentional act. In spite of these effects, use of valproate has the advantage of being easy to manage and it is also well tolerated in long term among patients. In severe intoxications, hemoperfusion or hemofiltration can be an effective means of quick elimination of the drug from the body.[8]

However, the circulating active molecule is the valproate ion, which is characterized by dose limited absorption, nonlinear plasma protein binding and multiple metabolic pathways of elimination.[9] The VPA concentrations are routinely monitored in serum and plasma due to the big variations in its absorption, distribution, biotransformation and excretion, to ensure the best regimen dosage and therapeutic effectiveness.[10] Therapeutic serum/plasma concentration of VPA is between 50-100 µg/mL during controlled therapy but its toxic serum/plasma concentration may reach 120-150 µg/mL.[11]

Hence, monitoring drug levels is particularly valuable in epilepsy for effective therapeutic drug management.[12]

The main methodologies used for VPA measurement in human serum are hereafter listed: immunoassay,[13] high performance liquid chromatography (HPLC) with ultraviolet (UV) detection[14] or fluorescence detection[15,16] or coupled with mass spectrometry (MS)[17,18] and capillary electrophoresis (CE) coupled with contactless conductivity detection (CD),[18,19] are the methods that were used for determination of VPA. Additionally, due to VPA volatility, GC is often used.[20,21] In order to prevent severe tailing of the fatty acid peak, on-column and pre-column derivatization has also been used.[22] Because VPA is a relatively strong organic acid, it does not chromatograph well on the standard nonpolar or slightly polar stationary phases routinely applied to clinical GLC determination of other anticonvulsant drugs unless first derivatized. VPA analysis in biological samples is difficult due to presence of proteins, salts and various organic compounds. Hence, sample preparation is crucial in drug analysis, which includes both analytes pre-concentration and sample cleanup.[23] In addition, VPA does not contain a nitrogen atom and is not detected by GLC nitrogen detectors. These requirements of special polar GLC columns or derivatization before GC with flame ionization detection are a few of the reasons that immunoassay is the most popular method to therapeutically monitor VPA.[10] However, GLC still offers advantages of specificity and low detection limits over immunoassay.

This paper presents a simple, sensitive, reliable, easy and precise method to perform GLC- flame ionization detector - routine VPA determination in plasma without derivatization and makes comparison of its results with those of immunoassay methods.

Materials and Methods

Blood samples

The full study protocol was approved by the Ethics Committee of Ain Shams University Hospital (ASUH). The study included 50 epileptic patients presently under the treatment of valproic acid from out patient's clinic, inpatients department and intensive care unit of Poison Control Center Cairo, ASUH from November 2015 to May 2016. Venous blood (5 mL) was withdrawn and collected in heparinized vacutainers, the separated plasma were divided into two aliquots. The first aliquot was used for the GC assay. The second aliquot was collected and stored at −20°C for immunoassay determinations.

Gas chromatography

Chemicals

VPA was obtained from Sigma (St. Louis, MO, USA). Chloroform, methanol (GC grade), octanoic acid, and hydrochloric

Figure 1. Chemical structure of valproic acid.

acid (analytical grade), were purchased from Merck (Darmstadt, Germany).

Instrumentation

Analyses were performed on a Dani Master (Italia) gas chromatograph (GC) equipped with a flame ionization detector (FID). Data acquisition and analyses were accomplished using the Clarity software package. Chromatography was performed isothermally at 135°C on a Gs-BP 100% dimethylpolysiloxane capillary column (10 m×0.53 mm ID, 2.65 μm film thickness, Supelco, Bellefonte, PA). Injection port and detector temperature were adjusted at 280°C. Gas flow rates were as follows: helium carrier gas, 25 mL/min; hydrogen, 70 mL/min, and air, 280 mL/min.

Standard solutions

Stock standard solutions (16 mg/mL) of VPA and (1 mg/mL) octanoic acid (internal standard; IS) were prepared in methanol and stored at −20°C. Working standard solutions for VPA were prepared in methanol producing 16, 8, 4, 2, 1, 0.5, 0.25, 0.125 and 0.0625 mg/mL.

Calibration curves and quantitation

Plasma standards for calibration curves were prepared by spiking different samples of 1 mLdrug-free plasma each with 20 μL of one of the above mentioned VPA working standards to produce 320, 160, 80, 40, 20, 10, 5, 2.5 and 1.25 μg/mL. They were shaked for 2 min. and then stored at least 15 min. at room temperature before use. The prepared calibration standards were pipetted into 4 mL polypropylene tubes and stored at -20°C pending analysis. In each run, a plasma blank sample was also analysed.

Extraction procedures

50 μL of IS were added to 200 μL of either calibrator, or sample. The solution was mixed lightly by hand, and 100 μL of 1N HCl were added and mixed lightly again. Chloroform (500 μL) was then added. The solution was vortex mixed for 15 second and centrifuged at 3400 rpm for 5 min. The organic layers were taken and added to another set of test tubes and 1 μL of the chloroform layer was injected into the GC.

Assay validation

The method specificity was evaluated by comparing the chromatograms obtained from the samples containing VPA and IS with those obtained from blank samples. A number of drugs were added to blank plasma and were analyzed according the current method for specificity validation. They include sodium diclofenac, sodium salicylate, phenytoin, Carbamazepine, caffeine and acetaminophen. Besides calibration standards, additional standards were prepared for the determination of intra-day (n=5) and inter-day (n=5) of the assay accuracy and precision. The absolute recovery (n=5) was calculated by comparing peak height obtained from prepared sample extracts with those found by the direct injection of drug solution made in methanol at the same concentration. The limit of quantification (LOQ) was estimated by analyzing VPA at low concentrations of the calibration curve. To determine the limit of detection (LOD), lower plasma concentrations than the lower end of the calibration curve were used. So, LOD can be defined as the concentration which caused a signal three times the noise. The accuracy of the procedure was determined by expressing the mean calculated concentration as a percentage of the spiked concentration.
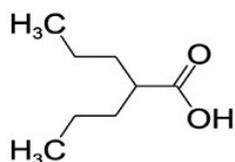
Immunoassay

VPA plasma level was performed by Homogeneous Immunoassays (HIA) on a cobas C 311 (Roch Hetachi), Japan. Calibration and controls were performed with the materials provided with the test kit. The calibration curve for the assay was constructed with six calibrator solutions supplied with the concentrations of 0, 12.5, 25, 50, 100, and 150 µg/mL. The obtained results were compared by plotting the correlation of valproic acid concentration with the two methods.

Results

Representative chromatograms of plasma spiked with 40 µg/mL VPA and a sample from an epileptic patient containing 77.7 µg/mL VPA with octanoic acid as internal standard are shown in Figure 2. The VPA and IS retention times were 1.8 and 2.3 min, respectively with relative retention which has been calculated by deviding the retention time of VPA by the retention time of IS equal to 0.78 min. No interfering peaks from the endogenous plasma components were observed either in the retention time of VPA or internal standard. None of the tested drugs interfered with the VPA assay. The calibration curve was linear over the concentration range of 5-320 µg/mL in human plasma with correlation coefficient greater than 0.999 as shown in Figure 3. The LOQ was 5 µg/mLand the LOD was 1.25 µg/mL. The values obtained for intra-day and inter-day precision and accuracy during the 5-day validation for plasma are shown in Table 1. The mean absolute recoveries for VPA and internal standard were 94.6 and 84.3%, respectively.

The correlation of the results obtained by HIA on a cobas C 311 *versus* the presented GLC method was 0.999 and illustrated in Figure 4. Linear regression demonstrated a good correlation between immunoassay results and those of the GLC method. There is a slightly positive bias difference between GLC and immunoassay values and hence it has no significant effect in the clinical interpretation of valproic acid results.

Discussion

Many methods have been reported for the determination of valproic acid in patients' serum samples.[24-28] However, studies focusing on direct determination of valproic acid concentration in patients with epilepsy are extremely limited. Direct determination of valproic acid is difficult and, in most of the previously described GC or HPLC methods, a derivatization step after deproteinization with acetonitrile[29-31] prior to chromatographic analysis is required. These procedures are susceptible to associate with poor reproducibility.[29] In addition, some of the analytic procedures may require specific detectors[30] that influence the cost of the procedures. Recently, high cost immunological assays have become attractive for routine clinical monitoring during chronic therapy. However, in some cases they are subjected to cross reactive interference problems as well.[28,32-34]

Free VPA is volatile with boiling point equal to 120°C thus the initial method for its quantitation in biological specimens used Conway microdiffusion.[35] However, for routine GLC serum determinations, less-complicated solvent extraction has been universally preferred in other reported procedures.[36,37] The aim of this study was to develop a selective, simple and sensitive quantitative ana-
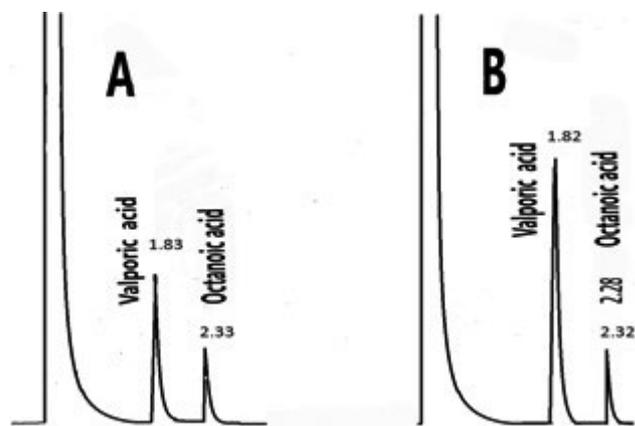
Figure 2. Chromatogram of plasma samples: A) plasma spiked with 40 µg/mL Valproic acid, B) a sample from an epileptic patient containing 77.7 µg/mL Valproic acid with octanoic acid as internal standard.

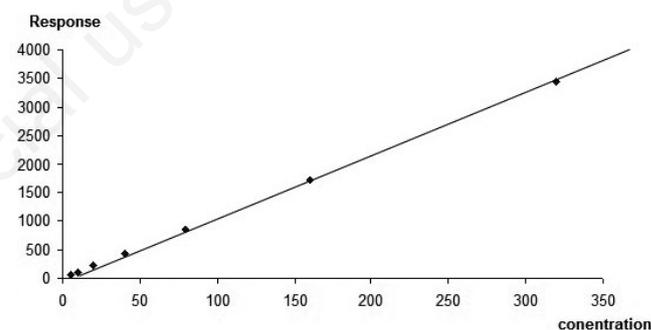**Figure 3. Linear calibration curve of Valproic acid at cocntrations from 10 to 320 µg/mL.**
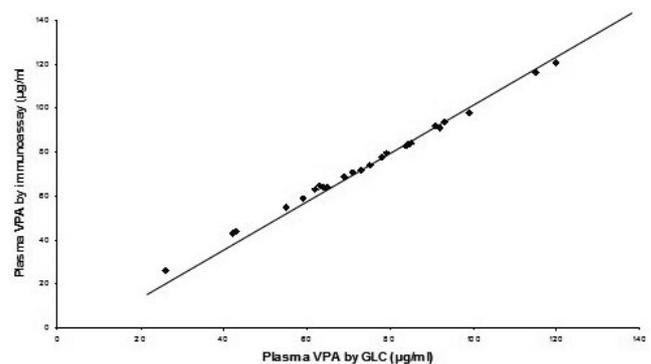

Figure 4. Linear regression correlation of Valproic acid results obtained by immunoassay (X-axis) *versus* the presented GLC method (Y-axis).

Table 1. Intra- and inter-day precision and accuracy and recovery data for plasma valproic acid.

Spiked concentration, µg/mL	Recovery %	Inter-day Mean±S.D.	Precision%	Acuracy%	Intra-day Mean±S.D.	Precision%	Acuracy%
5	95.6	5.1±0.16	3.5	2	4.8±0.14	3.5	−4
10	95.1	9.8±0.64	2.8	−2	10.5±0.32	5.8	5
80	94.5	81.5±1.32	2.1	1.8	82.3±1.41	2.1	2.8
160	94.1	158±3.12	3.4	−1.25	159±3.2	6.1	−0.6
320	93.8	328±6.82	2.5	2.5	324±5.9	4.1	1.2

lytical method for rapid determination of VPA in human plasma. Strong acid is generally added to the specimens for help in protein precipitation, followed by the addition of nonpolar extraction solvent such as; chloroform, dichloroethane or carbon disulfide and direct injection of a solvent aliquot onto the GC. The absence of a solvent evaporation step is essential to minimize the loss of the volatile free valproic acid. Like many previous studies, we found 1N HC1 and dichloroethane yielded acceptable recoveries and precision.

Initially, straight chain fatty acids such as caproic or caprylic acid were proposed as internal standards for VPA determinations. However, these compounds may be present in serum under abnormal metabolic conditions.[38] Octanoic acid has been successfully applied for many years as an internal standard without encountering significant interferences; thus, it was selected in the current study as an internal standard.

The chromatograms obtained after extraction of plasma spiked with VPA and a sample from an epileptic patient with octanoic acid as internal standard are shown in Figure 2 indicate that, the target compound and internal standard is separated well without any interferences of the biological extract. The VPA calibration curve was obtained by performing a linear regression analysis on spiked plasma samples (Figure 3). Good linearity was obtained with correlation coefficient (r >0.99). According to calibration calculations, the equation of the curve fitted to the calibration points were: Y=12.94 * X. All values for accuracy, recovery and precision were within recommended limits. In addition, the current method has been routinely used in our laboratory along two years with acceptable valproate results that obtained in external proficiency testing programs.

Conclusions

Although there are many GC methods in the previous research for VPA analysis in human plasma, a few of them could achieve sensitivity and selectivity similar to this method but with more complicated sample preparations. The new suggested method takes short time for VPA analysis and guarante the accurate results where the quantification of VPA recovery was 94.6%, hence this method is a quick and accurate technique for VPA determination in human plasma. The current innovative method with an optimized sample preparation achieved a good resolution and sensitive determination in human plasma. Furthermore, the suggested method validated a rapid and precise method with sufficient selectivity and sensitivity for determination of VPA in human plasma.

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