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Original Article

An Improved GC Method for Rapid Analysis of Valproic Acid in Human Plasma Without Derivatization

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Abstract

A simple, reproducible and rapid gas chromatographic method for precise determination of valproic acid (VPA) in human plasma has been developed. Total time for sample preparation and GC analysis is less than 45 min. After plasma protein precipitation, VPA was extracted into chloroform with suitable recovery. By using Stabilwax®-DA capillary GC column, a symmetrical gas chromatographic peak was obtained without the need for derivatization. The calibration curve was proved to be linear ($r^2 = 0.998$) in a wide concentration range (0.45-100 μg/ml). Inter-day and intra-day accuracy and precision of this method was investigated during the method validation and the method has good precision and accuracy. This method is highly reproducible with a limit of detection 150 ng/ml of VPA in human plasma and could be used in TDM and pharmacokinetic studies.

Keywords: Determination; Gas chromatography; Human plasma; Valproic acid.
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1. Introduction

Epilepsy is the most common serious neurological disorder affecting people of all ages. Statistics show that epilepsy affects 1% of the world population [1]. Valproic acid (2-propylpentanoic acid, VPA) has been widely used in the control of a variety of seizure types as a major antiepileptic drug [2, 3]. Determination of antiepileptic drugs in body fluids on a routine basis is important to clarify and control their therapeutic and toxic effects and to assess the patient's compliance to therapy [4-8].

Reported methods for analysis of VPA in serum and plasma include immunological techniques, chromatographic approaches [9-14]

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and techniques based upon capillary electrophoresis (CE) [15]. Most of the chromatographic methods require long sample preparation steps and prior derivatization [16]. Chromatography of the analytes without prior derivatization would significantly simplify the method and thus shorten the analysis time.

In the present study, we describe a simple and rapid method with improved sensitivity and reproducibility for sample preparation and GC analysis of VPA using a Stabilwax®-DA column with satisfactory performance.

2. Materials and methods

2.1. Chemicals

Valproic acid was obtained from Sigma (St. Louis, MO, USA). Perchloric acid (70%), chloroform and octanoic acid (analytical grade), methanol (HPLC grade) were purchased from Merck (Darmstadt, Germany). Laboratory prepared distilled water was used throughout the experiments. All reagents were used without further purification.

2.2. Apparatus

The gas chromatograph was a Hewlett-Packard (HP) model 6890 equipped with a

Table 1. Valproic acid in plasma, within-day assay precision, accuracy.

Spiked concentration ($\mu g.ml^{-1}$)	Observed concentration ($\mu g.ml^{-1}$)		Recovery[a]	Mean ($\mu g.ml^{-1}$)	SD ($\mu g.ml^{-1}$)	CV%	%RE[b]
0.45	Sample1	0.51	76±2%	0.468	0.041	8.70	+4.00%
	Sample2	0.48					
	Sample3	0.42					
	Sample4	0.50					
	Sample5	0.43					
1.00	Sample1	1.10	76±5%	1.040	0.057	5.48	+4.00%
	Sample2	1.06					
	Sample3	1.08					
	Sample4	0.97					
	Sample5	0.99					
10.00	Sample1	10.78	77±5%	10.720	0.552	5.15	+7.20%
	Sample2	11.19					
	Sample3	11.23					
	Sample4	9.89					
	Sample5	10.51					
20.00	Sample1	22.35	82±5%	20.730	1.193	5.75	+3.65%
	Sample2	21.17					
	Sample3	21.00					
	Sample4	19.74					
	Sample5	19.38					
50.00	Sample1	53.21	80±7%	51.440	1.617	3.14	+2.88%
	Sample2	49.62					
	Sample3	52.31					
	Sample4	52.26					
	Sample5	49.83					
100.00	Sample1	106.21	81±6%	103.760	4.161	4.01	+3.76%
	Sample2	108.28					
	Sample3	99.45					
	Sample4	99.22					
	Sample5	105.67					

[a]Average recovery calculated with 5 samples.
[b]%RE=[(mean value/theoretical value)-1]×100
The deviation of the mean from the nominal value serves as the measures of accuracy.

flame-ionization detector (FID). For instrumental control, data collection and processing GC- Chemstation software was employed. The column was a Stabilwax®-DA, 15 m×0.25 mm I.D.×0.25 m film thickness (RESTEK, Buckinghamshire,UK).

2.3. Standard solutions

Stock standard solutions (5 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 of VPA (1-500 μg/ml) were prepared from the stock standard solution in methanol and then aliquots of each of these solutions were added to the separated micro tubes .The spiked plasma samples were prepared freshly every day by adding 500 μl blank plasma to these tubes after solvent evaporation under nitrogen stream at the room temperature.

2.4. Processing of plasma samples

After addition of 20 μl of IS (1 mg/ml) and vortex for 1 min., 100 μl of perchloric acid (12%, w/w) and 250 μl of chloroform were added to each sample. The tubes were shaken for 5 min. vigorously and then centrifuged at 12000 rpm for 5 min. Then the top aqueous phase was removed and discarded. The chloroform layer was transferred to another

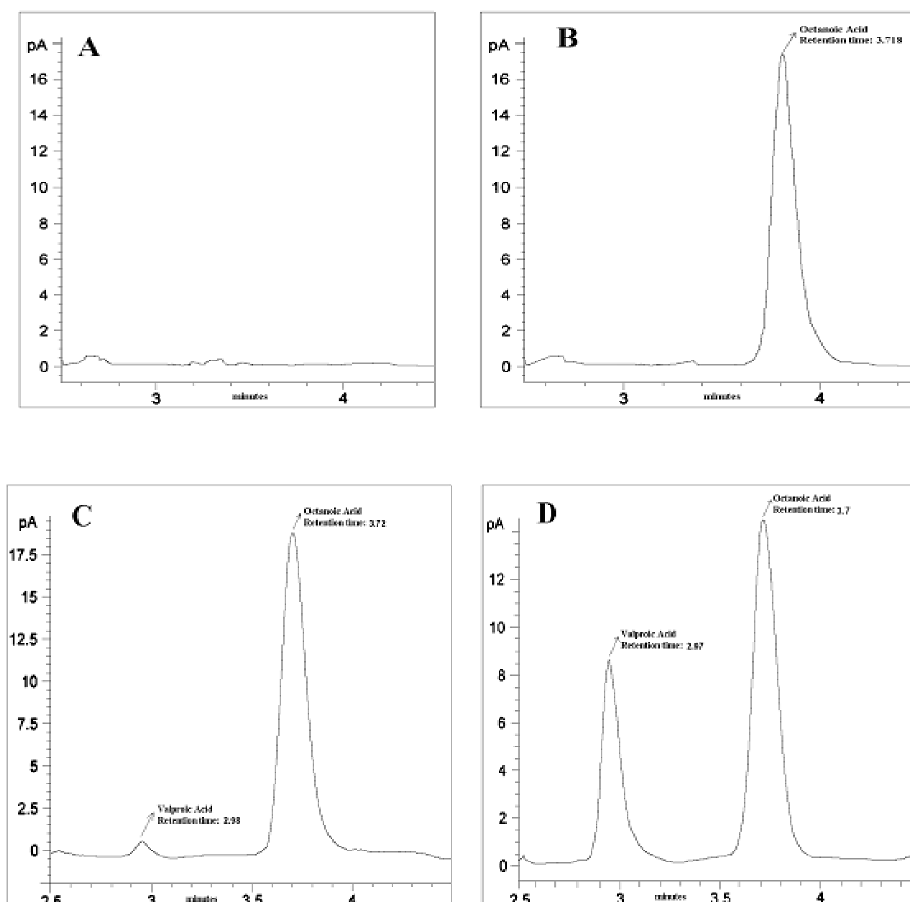

Figure 1: Chromatograms of A) plasma (blank), B) plasma spiked with 40 μg/ml octanoic acid, C) plasma spiked with 150 ng/ml VPA, and D) plasma spiked with 20 μg/ml VPA.

small tapered vial (*ca*. 200 µl was recovered). After evaporation of solvent under nitrogen stream and reconstitution with 50 µl of methanol, 3 µl of the sample was injected to GC inlet.

2.5. Gas chromatography conditions

Nitrogen was used as carrier gas at a flow rate of 3 ml/min. in a constant flow mode. The make up gas was also nitrogen. Splitless injection was used. The initial oven temperature was 100 °C (held for 2 min.) followed by an increase to 115 °C at a rate of 5 °C/min. (0 min. hold time) and at 0.6 °C/min. increase to 117 °C (1.5 min. hold time) and finally at 30 °C/min. to 220 °C (5 min. hold time). Optimum detector and injector temperature was 300 and 250 °C, respectively.

3. Results

3.1. Stability and recovery

Stability of stock standard solutions at +4 °C has been verified during a month. Stability of 10 µg/ml spiked plasma at -20 °C has been verified for VPA and octanoic acid during the sample storage period (two weeks). The extraction efficiency (recovery) was

Table 2. Valproic acid in plasma, between-day assay precision, accuracy.

Spiked concentration ($\mu g.ml^{-1}$)	Observed concentration ($\mu g.ml^{-1}$)		Recovery[a]	Mean ($\mu g.ml^{-1}$)	SD ($\mu g.ml^{-1}$)	CV%	%RE[b]
0.45	Day 1	0.41	75±7%	0.44	0.042	9.50	-2.22%
	Day 2	0.43					
	Day 3	0.46					
	Day 4	0.51					
	Day 5	0.41					
1.00	Day 1	1.16	75±6%	1.06	0.091	8.62	+6%
	Day 2	1.13					
	Day 3	0.96					
	Day 4	1.08					
	Day 5	0.97					
10.00	Day 1	11.14	78±5%	10.69	0.555	5.19	+6.9%
	Day 2	11.15					
	Day 3	10.77					
	Day 4	10.62					
	Day 5	9.79					
20.00	Day 1	20.15	80±6%	20.84	0.87	4.18	+4.2%
	Day 2	21.66					
	Day 3	19.98					
	Day 4	21.87					
	Day 5	20.54					
50.00	Day 1	52.33	81±6%	50.69	2.09	4.14	+1.38%
	Day 2	53.14					
	Day 3	50.72					
	Day 4	49.18					
	Day 5	48.11					
100.00	Day 1	106.22	82±8%	104.75	4.31	4.11	+4.75%
	Day 2	110.44					
	Day 3	99.62					
	Day 4	106.12					
	Day 5	101.35					

[a]Average recovery calculated with 5 samples.
[b]%RE=[(mean value/theoretical value)-1]×100
The deviation of the mean from the nominal value serves as the measures of accuracy.

determined for both VPA and octanoic acid by comparing peak areas from drug-free plasma spiked with known amounts of these compounds (in the range of concentrations of the calibration curves) and standard solutions in methanol injected directly into the GC inlet (Figure 1). Each sample was determined in triplicate. The results are presented in Tables 1 and 2. The extraction efficiencies for VPA and IS were 70-85%.

3.2. Linearity, precision and accuracy of the method

The method within and between-day precision in human plasma were assessed by performing five determinations per concentration of spiked plasma with VPA (at six levels) on the same and different days (n=5). The results are presented in Tables 1 and 2, respectively. Precision and accuracy were characterized by the RSD% and deviation from the nominal concentration, respectively. Linearity of the calibration curve was investigated in the 0.45-100 µg/ml concentration range, using spiked plasma samples. The calibration equation was calculated from the peak area values.

4. Discussion

So far, various GC methods have been used for determination of VPA in plasma, most of these methods have benefited from derivatization techniques to improve chromatographic characteristics and LOD of VPA but because of its multistep procedure and nature of chemical reactions, derivatization is not a good choice for rapid and robust quantitative analysis which are essential characteristics in TDM and clinical toxicological analysis. The aim of this study was to develop a selective and sensitive quantitative analytical method for rapid determination of VPA in human plasma. In order to determine the trace level of drugs, it is advantageous to eliminate any possible interference from samples. Using perchloric acid with chloroform to precipitate the plasma proteins and to extract the analyte in one step shorten the analysis time. By using a high polarity solvent such as methanol for reconstitution after previous non-polar solvent, we could gain a cleaner and more concentrate extract at the same time. After analysis of more than three hundred samples, there was no need to clean the sample inlet, and there was no significant increase in instrument noise. The chromatograms obtained after extraction of plasma blank, plasma spiked with 150 ng/ml, 20 µg/ml of VPA are shown in Figure 1. The chromatograms indicate that even in low levels, the target compound is separated from the interferences of the biological extract. The calibration curve for detection of VPA was obtained by performing a linear regression analysis on spiked plasma samples using the ratio of VPA to IS signal area. Good linearity was obtained with correlation coefficient of $r > 0.99$. According to calibration calculations, the equation of the curve fitted to the calibration points were: $Y= 0.026X-0.015$ ($r^2 = 0.998$). The detection limit of the method for VPA ranged from 120 to 180 ng/ml which was estimated at a signal-to-noise (S/N) ratio of 3. Compared with the previous published methods, the LOD of this method is better. The limit of quantification (LOQ) estimated by measuring the response of the calibration curve samples between run and defined as the concentration which yields an S/N equal to 10, was determined to be 450 ng/ml. The reproducibility of this method was represented by the percentage of the relative standard deviation (RSD) at each fortification level for VPA. The results show that the precision of the method was within 10% which is very satisfactory. The within-day reproducibility ranged from 3.14 to 8.70% and between-day reproducibility ranged from 4.11 to 9.50%.

5. Conclusion

Although there are many GC methods in

the literature for VPA analysis in human plasma, a few of them could achieve sensitivity and selectivity similar to this method but with more complicated sample preparation. By using these optimized sample preparation, column length and GC temperature programming, good resolution and sensitive determination was achieved in human plasma. We developed and validated a rapid and precise method with sufficient selectivity and sensitivity for determination of VPA in human plasma. The assay is based on fast sampling procedure which include protein precipitation with perchloric acid and liquid/liquid extraction with chloroform in one step, then reconstitution with methanol and finally direct injection to GC column without derivatization. The described method demonstrated to be suitable and sufficiently robust for TDM, toxicological and pharmacokinetic studies.

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