SIMPLE AND RAPID GAS-CHROMATOGRAPHIC METHOD FOR QUANTITATION OF TOTAL AND FREE VALPROIC ACID IN HUMAN SERUM

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Abstract- Valproic acid (VPA) is one of the mostly used antiepileptic drugs that may have some side effects so, it is highly recommended to evaluate its serum concentrations. The aim of this study was to develop a simple, fast and economic method using gas-chromatography equipped with flame ionization detector (GC/FID) and VPA analysis. To do this, 200 µl of serum was mixed with an aliquot of caproic acid (200 µL, methanolic solution) as internal standard and extracted by stepwise addition of hydrochloric acid and chloroform with slight agitation between each step. After centrifugation, 1.0 µl of the bottom layer was injected into a wide-bore nonpolar capillary column. Injectable samples for analysis of unbonded VPA were prepared by ultra filtration followed by solid phase extraction (SPE). Caproic acid and VPA were eluted after 1.5 min and 3.0 min, respectively (total GC run time about 3.2 minutes). This GC/FID method was linear over a range of 2.5-6400 µg/ml with the mean recovery of 92%. The intra- and inter-assay precision in the range of 25-100 µg/ml was 1.50-, 2.95, and 2.35-3.22%, respectively. The simplicity of sample preparation with no derivatization, short run-time and high sensitivity sufficient to detect low concentrations of the drug makes this method suitable for research as well as routine use.

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Key words: Valproic acid, gas-chromatography, serum

INTRODUCTION

Valproic acid (VPA), 2-propyl pentanoic acid, though indicated to control various forms of epilepsy and some non-epileptic disorders, may have adverse effects like osteopenia (1) and defective bone growth (2), spasmodic dysphonia (3), Fanconi syndrome (4), teratogenicity (5, 6), fatty liver (7), pancreatitis (8) and even obesity and reduced plasma antioxidant vitamins levels (9).

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As a histone deacylase (10), it may be also prescribed for non-epileptic disorders like migraine (11), glioblastoma multiform (12), and myelodysplastic syndromes (13, 14).

To minimize its adverse effects and maximize its therapeutic effects, the determination of its serum/plasma concentrations is crucial and necessitates the utilization of a rapid, simple, reliable and cost-effective test for therapeutic drug monitoring (15).

One of the known methods which can determine serum concentration of VPA is gas-chromatographic (GC) methods (16-21) and one of the most critical issues for GC quantitation of VPA is the selection of chromatographic column. It has been observed in previous studies that VPA is a relatively strong

organic acid and can not chromatograph well on the standard nonpolar or slightly polar stationary phases routinely applied to clinical GC determination for other anticonvulsant drugs unless first derivatized (22, 23). Most methods are based on the separation with columns developed for free fatty acids or modified carbowaxes (24, 25). Recently, the Nukol (a wide bore column with a polar stationary phase) have been used for fast and reproducible free VPA determination (21). However, polar stationary phases are not properly used in routine clinical labs and they are not stable at high temperatures.

The aim of this study was to develop a simple, reliable, and cost effective GC method to determine serum VPA concentrations on a suitable GC column. Several GC analyses with flame ionization detection (FID) using different solvents as well as solid phase extraction (SPE) columns and various internal standards were evaluated. Most were found unsuitable for our purpose due to the breakdown of drug molecules or between-run imprecision, until the present modified method was developed (24).

MATERIALS AND METHODS

Chemicals

N-caproic acid, VPA, standard solutions and VPA powder were purchased from Sigma Chemicals (USA).

All other chemicals were chromatographic grade and obtained from Merck (Germany). Solid phase extraction (VacElut 24) and filtration systems (MPS 4010) were from Varian and Millipore Company (USA).

Apparatus

Gas chromatograph (Younglin, South Korea, Model M600 D); Detector: FID; Column: 10 m × 0.53 mm, a wide bore column (TRB-1, film thickness 2.65 µm, Teknokroam, Spain).

Preparation of standard solutions

The amount of 1152 mg of VPA equivalent to 1000 mg of free VPA was dissolved in double distilled water made 100 ml (10000 mg/L) and then diluted to different concentrations and validated by a standard solution purchased from Sigma Chemicals and the one from Syva (USA) available in VPA EMIT kits. The internal standard was prepared by mixing of 10 µl of a pure caproic acid in 1.0 ml of methanol and brought to the total volume to 50 ml (186 mg/L).

Preparation of serum standards

Twenty µL of stock standard solution (10000 mg/L) was added to 1 ml of drug free pooled serum (200 mg/L), mixed vigorously, and then was diluted to 1/2, 1/4 and 1/8 by drug free serum to have concentrations of 100, 50 and 25 mg/L. These serum standards were prepared as a sample.

Total VPA analysis

Of each patients sera, calibrators and controls, 200 µl was transferred into extraction tubes, containing 200 µl HCl (1 mol/l) and then well mixed by an electrical vortex. Then 400 µl of the extraction mixture (equal volume of chloroform and methanol, containing internal standard) was added into each tube.

The contents of tubes were mixed thoroughly and centrifuged at 3000 g for 10 min at room temperature. Finally, 1.0 µl of bottom layer of the sample was injected into the gas chromatograph conditioned as follows: constant flow mode helium (carrier gas), air-hydrogen pressure for detection, flow rate: 20 ml/min, split ratio: 1.4; Constant flow mode helium (6.0 psi), Air-hydrogen flow for detection 350/25 ml/min, injector T = 200 ºC; Detector T = 290ºC; Initial oven temperature was set at 80 ºC and then raised to 100 ºC with the rate of 10 ºC/min, maintained for 1 min. Ramped at 5 ºC/min to 106 ºC and then held for 4 minutes.

Determination of free VPA

The GC injectable free fraction of the drug was prepared by ultrafiltration of 500 µl of serum by Amicon disk filters (cut off < 4000 Dal). An aliquot of 200 µl of clear bottom layer plus 200 µl internal standard solution (caproic acid 50 mg/L in H3PO4 0.7 M) was loaded to activate (column conditioning: two column volumes methanol, then 2 column volumes dist. water) BondElut C18 SPE columns (Varian, 100 mg, 1 ml) using VACELUT 24 (Varian). After washing with 2 volumes distilled

water free VPA was eluted by 200 µl acetone. One µl of eluted fraction was injected to GC with conditions that were same as total VPA.

Analytical recovery and accuracy

A recovery study was performed after spiking two quality control materials containing 15.5 and 38.0 µg/ml VPA with four different standard solutions prepared in our laboratory.

Method Comparison

To evaluate the GC method for serum VPA determination, serum samples of 83 patients with seizure of both sexes aged 27-80 years who were referred to our laboratory for therapeutic drug monitoring from different medical centers in Tehran, Iran, and were taking VPA were analyzed by both enzyme multiplied immunoassay (EMIT) (Syva Company) and our modified GC method.

Analysis of Data

The data were collected and subjected statistically to ANOVA test and Students *t* test. A *P* value less than 0.05 was considered significant.

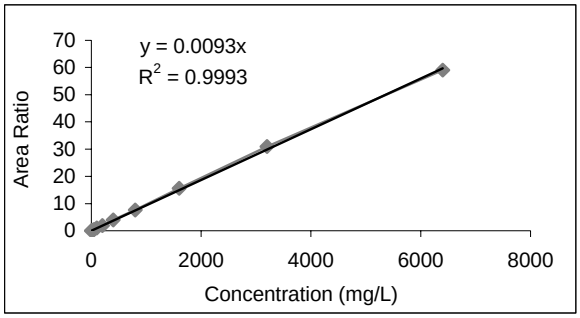
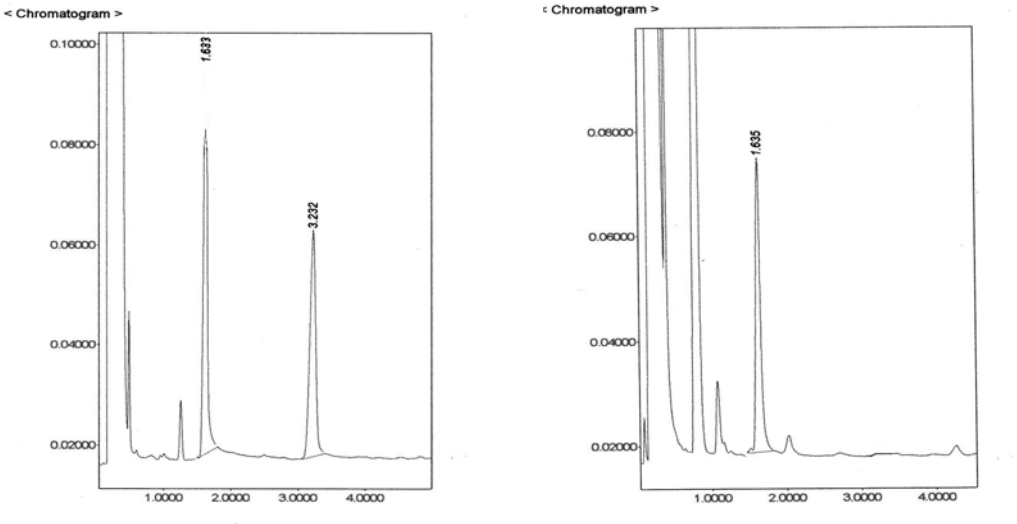


Fig. 1. Standard curve of valproic acid. Area ratio = VPA peak area/Internal STD peak area.

RESULTS

The method provided linear response over the concentrations of 2.5-6400 mg/L of VPA dissolved in methanol (Fig. 1). A calibration curve was prepared using 0-200 mg/L concentrations in drug-free serum. The absolute retention time of VPA was 3.2 min (Fig. 2).

Mean recoveries at concentrations of 25, 50, 100 and 200 µg/ml (11 replicates in 4 consecutive days) were 90.4%, 93.0%, 93.2%, and 91.3%, respectively.



(A) **(B)**

Fig. 2. A: Gas-chromatogram of valproic acid (3.22 min), (47 mg/L) and internal standard (1.63 min) in a patient serum. **B**: Gas chromatogram of a blank (drug free) serum with internal standard.

Table 1. Intra- and inter-assay variations of calibrators*

Concentration (mg/L)	Intra-assay coefficient variation	Inter-assay coefficient variation
25.0	1.49	2.61
50.0	2.95	2.35
100.0	1.92	3.22

* Data are given as percent.

Precision

Intra- and inter-assay variations for concentrations of 25, 50, 100 mg/L are shown in Table 1. The absolute retention time of VPA was 3.2 minutes.

Method Comparison

Results of VPA evaluation by both enzyme multiplied immunoassay (EMIT) (Syva Company) and our modified GC method are shown in Table 2. There was no significant difference between mean concentrations of serum VPA by both methods, as judged by Student *t* test and predetermined upper limit of significance $P < 0.05$. There was a significant correlation between GC method (y) and EMIT (x) for standard materials ($r = 0.97$, $P < 0.01$) (Fig. 3).

Day to day precision was determined by 20 replicates of quality control materials with two different drug concentrations using a newly extracted set of calibrators to calibrate the assay each day. The coefficient of variation (CV) at the concentrations of 50-150 mg/L was 3.0-5.2%.

DISCUSSION

The method provided linear response over the concentrations of 2.5-6400 mg/L of VPA dissolved in methanol (Fig. 1). This limit of analytical range was broader than the concentrations routinely found in our patients. VPA serum concentrations following therapeutic administration are typically from 50 to

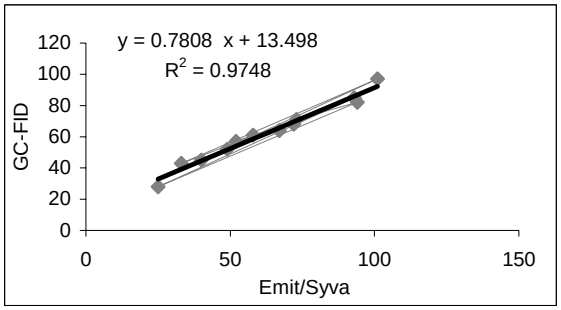


Fig. 3. Correlation between gas-chromatography and enzyme multiplied immunoassay for different concentrations of valproic acid standard solution.

100 mg/L (24). TRB-1 (HP-1) is a non-polar fused silica with 100% poly dimethylsiloxane and has high thermal stability and properly useful at general use. The short analysis time (4 min) and good resolution between peaks could be attained when we selected this column for VPA determinations. Participation in proficiency testing programs of "Lab quality", Finland, could provide a continual systematic means of assessing our analytical performance. The blind quality control programs provided four blood samples for analysis on quarterly basis. The results were comparable with those of more than 100 participant laboratories.

In conclusion, the significance of the method is the simplicity of sample preparation without need for drivitization, short run time of chromatographic step and good sensitivity which is enough for free drug analysis (25-27). Our data shows that this method can be well used for routine as well as research purposes.

Acknowledgement

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Conflict of interests

We have no conflict of interests.

Table 2. Comparison of serum valproic acid concentrations by GC and EMIT in 83 patients*

Sex	Age (years)	Mean Conc. (mg/L) by GC	Mean Conc. (mg/L) by EMIT	Difference
Male (n=59)	55.3 ± 12.4 (27-80)	57.9 ± 24.9 (17 - 111)	60 ± 25.7 (18 - 114)	NS
Female (n=24)	54.5 ± 8.3 (50-71)	65.4 ± 24.6 (18 - 121)	67.7 ± 25.3 (18 - 124)	NS

* Data are expressed as mean ± standard deviation. Numbers in parentheses show minimum and maximum values in each group.
Abbreviations: EMIT, enzyme-multiplied immunoassay; GC, gas-chromatography; NS, non-significant.

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