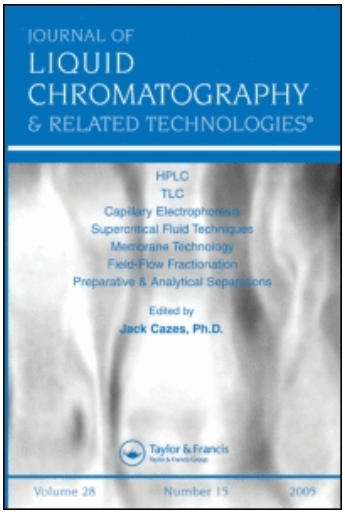

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HPLC DETERMINATION OF VALPROIC ACID IN HUMAN SERUM USING ULTRAVIOLET DETECTION

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ABSTRACT

A sensitive HPLC method with minimal sample preparation and good reproducibility for the determination of valproic acid in serum is described. Serum samples were precipitated using acetonitrile containing diazepam as the internal standard. Chromatography was performed on a Hewlett Packard model 1090 equipped with an octadecylsilane column and a Beckman model 163 variable wavelength detector. The drug and internal standard were eluted isocratically using a mobile phase consisting of 0.01M sodium phosphate monobasic solution, pH 2.3 and acetonitrile (63:37 v/v) followed by a gradient to flush the column before the next sample injection. The flow rate was 2.5 mL/min, the injection volume was 25 µL and the effluent was monitored at 210 nm. The serum standard curve was linear from 2.5-200.0 µg/mL with a correlation coefficient of 0.9994. Day-to-day precision for quality control samples (10.0, 25.0, 75.0 µg/mL serum) ranged from 5.6-9.6% CV. Possible interferences from other drugs which might be administered concurrently were studied. The method has been applied to the analysis of human serum samples.

INTRODUCTION

Valproic acid (2-propylpentanoic acid) is an antiepileptic agent used to control several types of seizures. Structurally unrelated to other anticonvulsant drugs, valproic acid has been approved for use in the United States since 1978. It is commonly used in conjunction with other drugs and has been associated with both increased and decreased plasma concentrations and side effects of the concomitant medication (1).

Several methods are described in the literature for the analysis of serum valproic acid. These include gas chromatography, fluorescent and enzyme immunoassay, and high-performance liquid chromatography. The GC methods utilize direct injection of a deproteinized sample, or extraction with or without derivatization (2-14). Immunoassay methods have also been employed to analyze valproic acid in serum (15-19). These techniques require a small sample volume and are rapid but have some cross-reactivity to the metabolites of valproic acid, and the reagents are relatively expensive.

Recently HPLC methods for the determination of valproic acid in serum have become available (20-28). Most of these utilize a solvent extraction step followed by derivatization to enhance absorbance in the ultraviolet (21-22,24-26,28) or visible (20,27) region. One method uses direct serum injection coupled with mass-spectrometric detection (23). All the HPLC methods suffer from certain limitations including 1) the need for special sample extraction procedures which minimize the loss of valproic acid due to volatilization, 2) the use of a derivatization step to enhance the UV or visible absorbance, 3) the lack of an internal standard, 4) the use of a highly flammable or toxic extraction solvent and 5) a poor limit of determination.

This paper describes a sensitive method for valproic acid determination which does not suffer from the above limitations. Sample preparation time and valproic acid volatilization are minimized by using acetonitrile (containing internal standard) to precipitate the serum proteins and then directly injecting an aliquot of the supernatant. The need for derivatization has been eliminated since the use of a high efficiency octadecylsilane (ODS) column in reversed phase mode coupled with

monitoring of the UV absorbance at 210 nm afforded a 2.5 µg/mL limit of determination.

MATERIALS AND METHODS

Instrumentation:

Chromatography was performed with a Hewlett Packard model 1090 liquid chromatograph equipped with a Beckman model 163 variable wavelength detector, an autosampler, and an octadecylsilane column, 4.6 mm i.d. x 15 cm long, packed with 5 micron spherical material (Zorbax ODS, DuPont). The eluent was monitored at 210 nm and the response was recorded on a 3392A Hewlett Packard integrator. The degassed mobile phase was pumped through the column at 2.5 mL/min, and the column compartment was maintained at 40°C.

Reagents:

Valproic acid and diazepam were obtained from Interchem Corporation and Hoffmann-La Roche Incorporated, respectively. Sodium phosphate monobasic (reagent grade), phosphoric acid (85%, HPLC grade) and methanol (HPLC grade) were obtained from Fisher Scientific Co. Acetonitrile (HPLC grade) was obtained from J.T. Baker Chemical Co.

Drug Solutions:

A 1000.0 µg/mL stock solution of valproic acid was prepared in methanol.

Internal Standard (IS) Solutions:

A 100.0 µg/mL stock solution of diazepam was prepared in methanol. The precipitation solution consisted of 250 ng diazepam/ mL acetonitrile.

Mobile Phase:

Sodium phosphate monobasic solution (0.01 M) was prepared in deionized distilled water, and the pH was adjusted to 2.3 with phosphoric acid. The mobile phase for the assay consisted of 63% buffer and 37% acetonitrile (v/v) to elute the valproic acid and internal standard. The percentage of acetonitrile was then gradually increased to 60% where it was maintained for 1.5 minutes to flush the column. The gradient was then

reversed and the column allowed to equilibrate at 37% acetonitrile for 3 minutes prior to the next sample injection.

Preparation of Serum Standards:

To a known volume of drug-free serum, an aliquot of valproic acid solution was added to prepare standards containing 2.5-200.0 µg valproic acid/mL serum. A 250 µL aliquot of spiked serum was then placed into a 10 x 75 mm pyrex tube, and the proteins were precipitated with 500 µL acetonitrile containing 125 ng of the internal standard. The tubes were vortexed for 15 seconds and centrifuged for 10 minutes at 1400xg. An aliquot of the supernatant was transferred to a glass microvial before injection of 25 µL into the chromatograph.

Quantification:

Standard curves for serum were constructed utilizing four replicates at each concentration (2.5-200.0 µg valproic acid/mL serum). The peak heights were integrated and the ratios of valproic acid to internal standard were plotted against concentration (µg drug/mL serum).

Recovery:

Spiked samples containing known concentrations of valproic acid (2.5-200.0 µg/mL) in serum or water were carried through the analysis procedure in triplicate. The peak height ratios obtained from the serum samples were compared to the ratios obtained from the equivalent water samples to estimate percent recovery after serum protein precipitation.

Patient Samples:

Serum samples obtained from patients receiving valproic acid were analyzed using the procedure described above for serum standards. The amount of drug in the patient samples was calculated utilizing a standard curve prepared daily.

Quality Control (QC) Samples:

Drug-free serum was spiked with known concentrations of a valproic acid solution prepared in methanol. Three quality control levels (10.0, 25.0, and 75.0 µg/mL serum) were prepared, aliquoted, and stored at -20°C until needed for use. After the samples were brought to room temperature, the samples were carried through the serum assay. The amount of drug found in the QC samples was calculated by comparison to a standard curve prepared daily.

Interferences:

The possible interference of normal serum constituents was tested by the analysis of drug-free serum samples. The interference of other drugs was tested by direct injection of aqueous or methanolic drug solutions and by the analysis of three types of commercially prepared therapeutic drug monitoring (TDM) sera: a lyophilized quality control obtained from Ortho Diagnostic Systems (Raritan,NJ); a performance check sample obtained from Ames Division Miles Laboratories, Inc. (Elkhart,IN); and a therapeutic drug monitoring proficiency survey sample obtained from the College of American Pathologists (CAP)(Skokie,IL).

Comparison of Sample Assay Values by HPLC Method and FIA Analysis:

Serum samples obtained from a volunteer who received a single 500 mg oral valproic acid dose were analyzed by HPLC and by fluorescence immunoassay (FIA) (Ames Optimate, Elkhart, IN) for comparison of sample assay values.

RESULTS AND DISCUSSION

In reviewing the analytical GC and HPLC literature for valproic acid the following factors were considered: 1) the choice of method for determination, 2) the choice of chromatographic conditions and detection, 3) the choice of an internal standard, 4) the potential loss of valproic acid via volatilization, 5) the choice of extraction or protein precipitation for sample preparation, 6) the need for a derivatization step, and 7) the limit of detection and linearity range required for the determination of valproic acid in serum.

The method for the analysis of valproic acid described in this paper is an HPLC procedure which utilizes UV detection and eliminates sample extraction and derivatization steps.

The chromatographic conditions chosen for the valproic acid assay were selected to give good separation of valproic acid and the internal standard from endogenous serum peaks. The use of a high efficiency 5 micron particle size octadecylsilane column in a reversed phase mode with an isocratic mobile phase of 0.01M sodium phosphate monobasic solution,

pH 2.3 and acetonitrile (63:37; v/v) produced excellent separation of valproic acid from the internal standard. After the drugs were eluted from the column the percentage of acetonitrile was increased to 60% for 1.5 minutes in order to flush the column. The use of a gradient allowed baseline resolution of valproic acid and the internal standard from endogenous serum peaks during repetitive sample injections. Typical chromatograms are found in Figure 1. The retention times for valproic acid and the internal standard were 5.1 and 7.7 minutes respectively. A total analysis time of approximately 14 minutes per sample was necessary to insure elution of endogenous serum peaks and to allow column equilibration after the gradient flush.

The use of a Beckman 163 variable wavelength detector set at 210 nm (the maximum absorbance wavelength for valproic acid) gave good sensitivity for the detection of valproic acid and the internal standard. The limit of detection for the method based on a signal to noise ratio of 3 was approximately 1.5 µg valproic acid/mL serum. This level is lower or equivalent to the reported sensitivities of the published HPLC methods (20-28).

Precipitation of serum protein with acetonitrile containing the internal standard was chosen for the current method. This simple procedure eliminates sample extraction and derivatization steps and allows the rapid handling of a large number of serum samples. The use of acetonitrile for precipitation of serum proteins has the added advantages of limiting the loss of valproic acid due to volatilization without compromising the limit of detection and of being the organic modifier in the mobile phase. The overall percent recovery from serum samples containg 2.5-200.0 µg valproic acid/mL serum was 74.6% using the described method. The loss of drug during protein precipitation may be due to protein binding (1,17-18) and co-precipitation of valproic acid with protein.

The addition of internal standard to the precipitation solution allowed the ratio of the peak height of drug to the peak height of internal standard to be calculated at each serum concentration. Statistical analysis of the data by linear regression indicated excellent linearity and reproducibility in the range of 2.5-200.0 µg/mL serum (Table 1). The linear range is applicable to monitoring therapeutic valproic acid levels as well

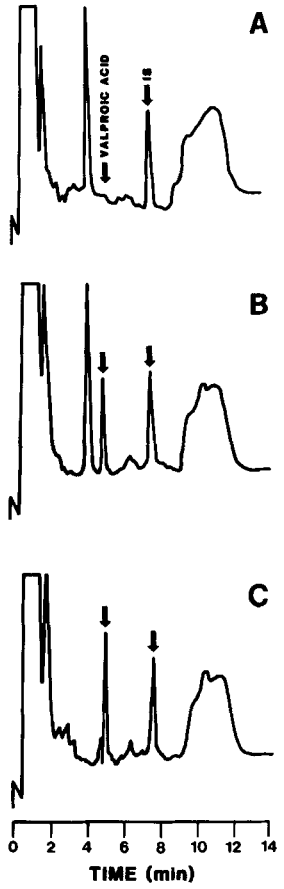

Figure 1. Typical Valproic Acid HPLC Chromatograms. Key: A, 0.0 µg/mL Serum Standard; B, 50.0 µg/mL Serum Standard; and C, Patient Serum Sample (Equivalent to 71.9 µg/mL Serum).

TABLE 1. LINEARITY AND PRECISION OF THE VALPROIC ACID SERUM ASSAY

Valproic Acid Added (µg/mL serum)	Valproic Acid Found (µg/mL serum)*	CV (%)
2.5	3.7 ± 0.3	8.1
5.0	5.2 ± 0.1	1.9
10.0	9.6 ± 0.5	5.2
25.0	23.7 ± 0.5	2.1
50.0	47.1 ± 0.7	1.5
75.0	72.6 ± 2.8	3.9
100.0	98.7 ± 1.6	1.6
125.0	123.9 ± 0.5	0.4
150.0	149.8 ± 2.6	1.7
200.0	203.4 ± 3.9	1.9

N=4 replicates at each concentration
Overall CV= 2.8%
Correlation Coefficient = 0.9994
*mean ± standard deviation

as to the analysis of bioavailability study samples. The 2.5 µg/mL limit of determination based on a CV of less than 10% is competitive with the concentrations listed for other HPLC methods.

This assay has been applied to the analysis of serum samples obtained from patients taking valproic acid. Quality control samples were analyzed each day with the patient samples, and the method showed very good day-to-day precision (Table 2). The quality control sample results demonstrate good stability of valproic acid in frozen serum samples as well as the accuracy of the method.

No interference from normal serum constituents was observed after precipitation and chromatography (Fig. 1A). Three commercially prepared TDM serum products (obtained from Ortho, Ames and CAP) containing a mixture of therapeutic drugs were carried through the assay. The results obtained by this HPLC procedure compared well with the overall mean values obtained by interlaboratory analyses using gas chromatography and immunoassay methods to determine valproic acid (Table 3). These results indicate that the HPLC method is specific and accurate since little

TABLE 2. REPRODUCIBILITY OF THE VALPROIC ACID SERUM ASSAY

Quality Control Concentration (μg/mL serum)	Concentration Found (μg/mL serum)*	CV (%)
10.0	9.8 ± 0.9	9.6
25.0	25.7 ± 1.4	5.4
75.0	77.1 ± 4.3	5.6

N=13 days for day-to-day precision
Overall CV=6.9%
*mean ± standard deviation

TABLE 3. VALPROIC ACID CONCENTRATIONS FOUND IN THREE COMMERCIAL TDM SERUM PRODUCTS

Assay Method	TDM Serum Products		
	Ortho	Ames	CAP
HPLC (μg/mL serum)[a]	144.6	123.2	41.9
Interlaboratory (μg/mL serum)[b,c]	130. ± 10.	129.± 10.	40.2 ± 3.4
SDI[d]	1.5	-0.6	0.5
Number of drugs in serum	27	13	8

[a]
[b]proposed HPLC method
[c]overall mean ± standard deviation
[d]gas chromatography and immunoassay methods
SDI= Standard Deviation Interval

$$= \frac{\text{(HPLC result) - (Interlaboratory analyses mean)}}{\text{standard deviation of interlaboratory results}}$$

interference from the other drugs in the quality control and performance survey samples was displayed. Chromatography of methanolic solutions of the common antiepileptics (carbamazepine, ethosuximide, phenobarbital, phenytoin and primidone) indicated that they all eluted early and were well separated from valproic acid.

A typical kinetic profile of serum samples containing valproic acid obtained from a volunteer receiving a single 500 mg oral dose and assayed by the HPLC method is shown in Figure 2. These samples were also analyzed using the Ames Optimate fluorescent immunoassay method.

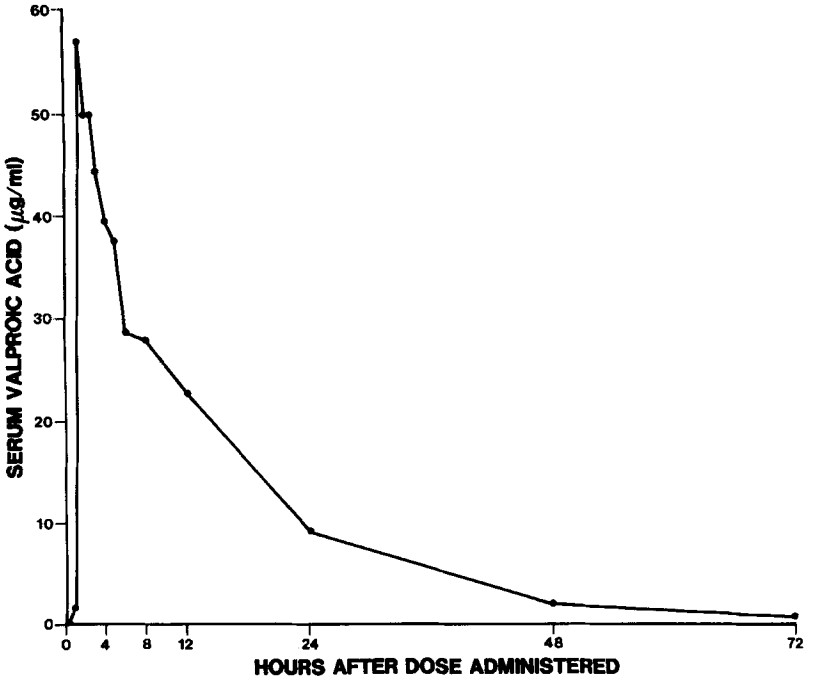

Figure 2. Serum Concentration of Valproic Acid After Administration of a Single 500 mg Oral Dose.

Comparison of the sample results obtained by these two methods resulted in a correlation coefficient of 0.9913. The presence of valproic acid metabolites in the volunteer samples which could cross-react and interfere with the FIA determination might contribute to the variation between the results of the two methods. Two chromatographic peaks were seen at 3.4 and 4.7 minutes in patient samples and may be valproic acid metabolites (Figure 1C). These peaks were present in all patient samples and volunteer samples tested except for the predose samples.

CONCLUSIONS

The HPLC method for serum valproic acid determination presented in this paper has several advantages over the procedures presently described

in the literature. The method uses a simple acetonitrile protein precipitation step instead of extraction and derivatization, and yet has equivalent sensitivity to other procedures. The method has excellent linearity and reproducibility and takes into consideration the volatile nature of valproic acid.

The specificity, simplicity, reproducibility and linearity for this assay make it applicable for determining valproic acid levels in bioavailability study samples, or for monitoring therapeutic valproic acid levels in patients. Results obtained by this HPLC method compare favorably with other methods, and the method has been used to analyze several hundred human serum samples in our laboratory.

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