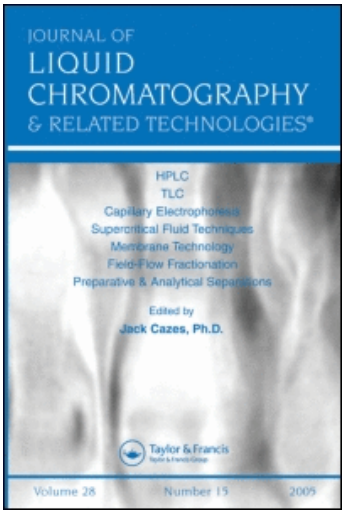

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Liquid Chromatographic Determination of Valproic Acid in Human Serum

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LIQUID CHROMATOGRAPHIC DETERMINATION OF VALPROIC ACID IN HUMAN SERUM

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ABSTRACT

The concentration of the antiepilepsy drug valproic acid (2-propylpentanoic acid) was determined in both a processed freeze dried human serum material and patient serum samples obtained from a clinical laboratory. The freeze dried material is being issued by the National Bureau of Standards as Standard Reference Material 1599. The analytical procedure developed involves organic extraction of valproic acid and an internal standard (cyclohexanecarboxylic acid) from the serum matrix; derivatization of the carboxylic acids to phenacyl esters; measurement of the analyte and internal standard species by reversed-phase high performance liquid chromatography. The results obtained on both types of samples compare favorably with results obtained using more conventional gas chromatographic approaches.

INTRODUCTION

In the late 1970's valproic acid (2-propylpentanoic acid) became an important drug in the treatment of epilepsy (1,2), and consequently, the need arose for analytical procedures capable of providing accurate and precise measurements of the drug in a serum matrix. The work of Durst and Grushka (3,4) in 1975 showed that phenacyl esters of carboxylic acids can be easily prepared through the use of a crown ether catalyst. The phenacyl ester

derivative can then be chromatographed by reversed-phase high performance liquid chromatography (HPLC).

Schmidt and Slavin (5) first applied the techniques of phenacylation and HPLC to the analysis of valproic acid in 1978. They only worked with organic solutions of pure compounds, however, and did not address the problems associated with the analysis of the drug in a biological matrix. The potential of applying the method of phenacylation to the analysis of valproic acid in serum has, for the most part, been over-looked. Although many procedures for the analysis of valproic acid have been published since 1975, the predominant scheme involves a liquid-liquid extraction of the free acid and an internal standard, and analysis by gas chromatography (GC) (6-11).

One advantage of GC procedures is that they permit the direct analysis of valproic acid whereas HPLC procedures require a derivatization step such as phenacylation to enhance the UV absorbance. A disadvantage of working with the free acid is that extreme care must be taken to avoid losses due to volatilization when concentrating the sample extract (6,7). Several investigators have avoided the need to concentrate the extract by utilizing a back extraction technique to remove interferences (8,9), while others have used micro scale extraction followed by the direct injection of an aliquot of the extract (10). Fellenberg and Polland (11) addressed this problem by extracting with a nonpolar solvent (n-heptane) which yielded a cleaner extract. The detection limit for each of these procedures is thus a function of its ability to produce a clean sample extract. An additional problem associated with GC procedures is that the free acid does not chromatograph well on most conventional GC packing materials (7). Severe peak tailing is often observed unless special packings capable of handling polar compounds are used. Finally, the heavily loaded packings commonly used for polar compounds often produce excessive column bleed and deteriorate quickly (12).

Our interest in the HPLC determination of valproic acid arose from the need for an accurate and precise method for the certification of three concentration levels of this drug in a freeze-dried human serum matrix that will be issued by the National Bureau of Standards (NBS) as Standard Reference Material (SRM) 1599.

The human serum matrix is a processed pooled serum lot specially prepared for NBS. Although not discussed in this paper, SRM 1599 also contains three concentration levels of carbamazepine, another antiepilepsy drug. This drug was determined by separate analytical procedures and its presence in the serum matrix did not interfere with the analysis of valproic acid.

The certification of an SRM requires the use of a "definitive" method, where the analyst has complete knowledge of all sources of error, or at least two independent analytical procedures. In this paper we will describe the modifications to the procedure of Schmidt and Slavin that we found necessary to provide us with one of the two procedures required for certification. We found this to be an accurate and precise method for the analysis of valproic acid in both a freeze-dried serum matrix and in serum samples obtained from a clinical laboratory.

MATERIALS AND METHODS

Reagents

HPLC grade methanol, acetonitrile, water, and methylene chloride were used. The derivatizing reagent was Phenacyl-8 obtained from the Pierce Chemical Co.*, Rockford, Il., and

*Certain commercial equipment, instruments, or materials are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Bureau of Standards, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

contained 0.1 mmol/mL phenacylbromide and 0.005 mmol/mL crown ether in acetonitrile. The stock Phenacyl-8 was diluted 1-5 with acetonitrile prior to use. The phosphoric acid used in this work was reagent grade. Valproic acid was purchased from Saber Laboratories, Morton Grove, Il., and cyclohexane carboxylic acid was obtained from Eastman Kodak Co., Rochester, N.Y. The reaction vessels and sealing tool used in this work can be obtained from most chromatographic supply houses.

Processing of Human Serum for Use as SRM 1599

The serum base was procured from Seraplex, Inc. (Arcadia, CA.). Following procurement of this serum, the material was further processed at a local biological production plant where it was pooled into a single lot (approximately 24 L) and stirred to achieve homogeneity. The serum was then passed through depth and membrane filters, having a final pore size of 0.22 μm to achieve sterility, into four sterile containers, each marked for a volume of 4 L. Samples of the filtrate were tested by HPLC for suitability and freedom from interfering peaks prior to spiking with valproic acid and carbamazepine.

One of the four containers was designated as Blank Serum. The three remaining containers were spiked with valproic acid and carbamazepine to produce three concentration levels roughly corresponding to human sub-therapeutic, therapeutic, and toxic serum levels of the drugs. The three spiked containers were labeled low, medium, and high, respectively.

The contents of each container were dispensed in 5.00 mL aliquots into vials that were sequentially numbered and appropriately labeled. The contents of each vial was then freeze-dried and the vial stoppered under nitrogen.

Instrumentation

The HPLC system used in this work consisted of a variable volume injector, auto-sampling accessory, two reciprocating

diaphragm pumps, variable wavelength UV detector, and an integrator-printer/plotter. The column used in this work was monomeric octadecylsilane (C-18).

SAMPLE PREPARATION

SRM 1599

Samples of freeze-dried human serum (SRM 1599) were reconstituted with 5.00 mL of water. The samples were allowed to remain at room temperature for 1 h with occasional rotation (by hand) to ensure complete dissolution.

A 1.00 mL aliquot of each sample was placed in a 15 mL screw-top vial. Known amounts of cyclohexane carboxylic acid were added to each vial to serve as an internal standard. The serum aliquot was acidified by adding approximately 100 µL of concentrated phosphoric acid, then extracted with 2.0 mL of methylene chloride. The extraction step was accomplished by placing the capped vials on a wrist-action automatic shaker for 30 min. The vials were than centrifuged for 10 min at 2500 rpm to separate the aqueous and organic layers.

Approximately 0.5 mL of the sample extract was placed in a 1 mL reaction vessel with 0.5 mL of the derivatizing reagent solution and approximately 0.5 mg of sodium bicarbonate. The vessels were sealed and heated at 75 °C for 2 h. Upon completion of the reaction period, each vessel was opened and its contents concentrated with nitrogen purge to approximately 0.5 mL. The vessels were then filled to ∿1 mL with acetonitrile before aliquots were taken for analyses by HPLC.

Figure 1 shows the reaction scheme which was used to produce the phenacyl ester derivatives of valproic acid and cyclohexane carboxylic acid. A 30 min. gradient from 40 to 80 percent acetonitrile in water was used for analyses.

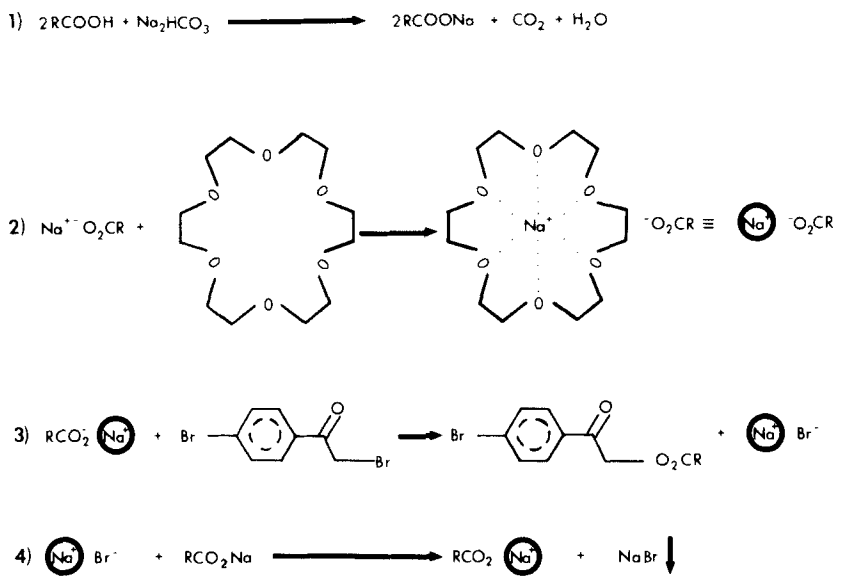

FIGURE 1. Phenacylation of valproic acid. R = C_7H_{15}.

Patient Serum Samples

Essentially the same sample preparation procedure was used for both the processed freeze dried SRM sample and the samples of human sera obtained from the University of North Carolina. The modifications that were made were as follows: 0.1 mL instead of 0.5 mL of derivatizing reagent solution was used; the incubation time was reduced from 2 h to 0.5 h.

The patient serum samples were analyzed under isocratic conditions, using a 65 percent acetonitrile in water mobile phase. The HPLC analysis time was 10 min.

Preparation of Standards

Stock solutions of valproic acid and cyclohexane carboxylic acid (1 mg/mL level) were prepared by accurately weighing appro-

priate amounts of the crystalline material into 100 mL volumetric flasks and diluting to the mark with 10 percent methanol in water.

Working standards, appropriate to each sample concentration level, were prepared by adding aliquots of the stock solutions to blank serum. The working standards were then processed as described in the sample preparation section above.

Six working standards were prepared and processed with the samples from each concentration level. The standards ranged in concentration from 9-20, 50-80, and 120-185 μg/mL of valproic acid for the low, medium, and high concentration levels respectively. The internal standard, cyclohexane carboxylic acid, was added to samples and working standards to produce concentrations of 50 μg/mL for the low and medium concentration levels and 100 μg/mL for the high level.

RESULTS

Fifteen samples from each concentration level of SRM 1599 were analyzed in duplicate by reversed-phase HPLC. A typical chromatogram of a medium level sample is shown in figure 2. Figure 3 is a standard curve containing area response ratios (valproic acid to internal standard) versus the concentration of valproic acid in working standards ranging from 5-200 μg/mL. Although this curve appears linear over the entire concentration range investigated, we preferred to quantitate using calibration curves that covered the smaller ranges mentioned earlier.

The mean value obtained from the HPLC analysis of each concentration level of SRM 1599 is shown in table 1 along with results obtained using two additional methods of analysis. The NBS-GC values were obtained during the course of certifying this processed serum as an SRM. The NC-GC values were provided by Dr. Ken Dudley, University of North Carolina School of Medicine.

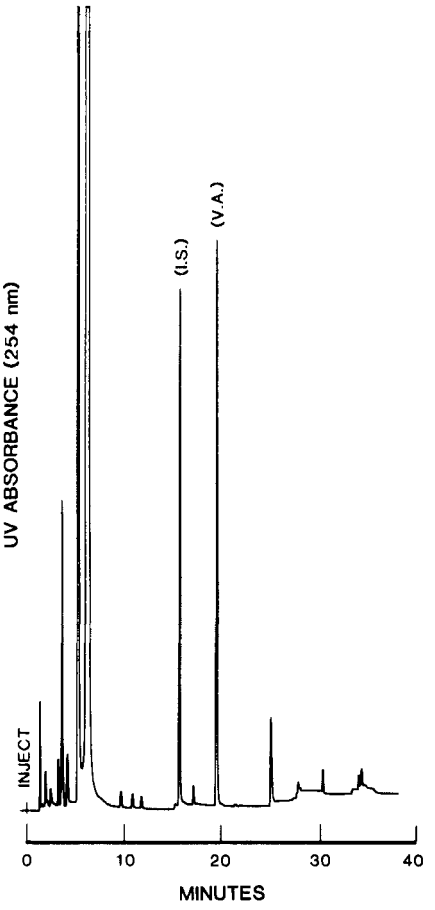

FIGURE 2. HPLC chromatogram of SRM 1599, medium level sample. I.S. = cyclohexane carboxylic acid, V.A. = valproic acid.

TABLE 1

Determination of Valproic Acid in SRM 1599 by Three Methods of Analysis

	Concentration (µg/mL)		
	Low Level	Medium Level	High Level
NBS-HPLC	14.6 ± 0.3[a]	67.1 ± 0.9	145 ± 1
NBS-GC	14.3 ± 0.1	71.2 ± 0.4	141 ± 1
NC-GC	14.7 ± 0.4	67.5 ± 1.5	141 ± 1

[a]Uncertainties reported represent the standard deviation of the mean.

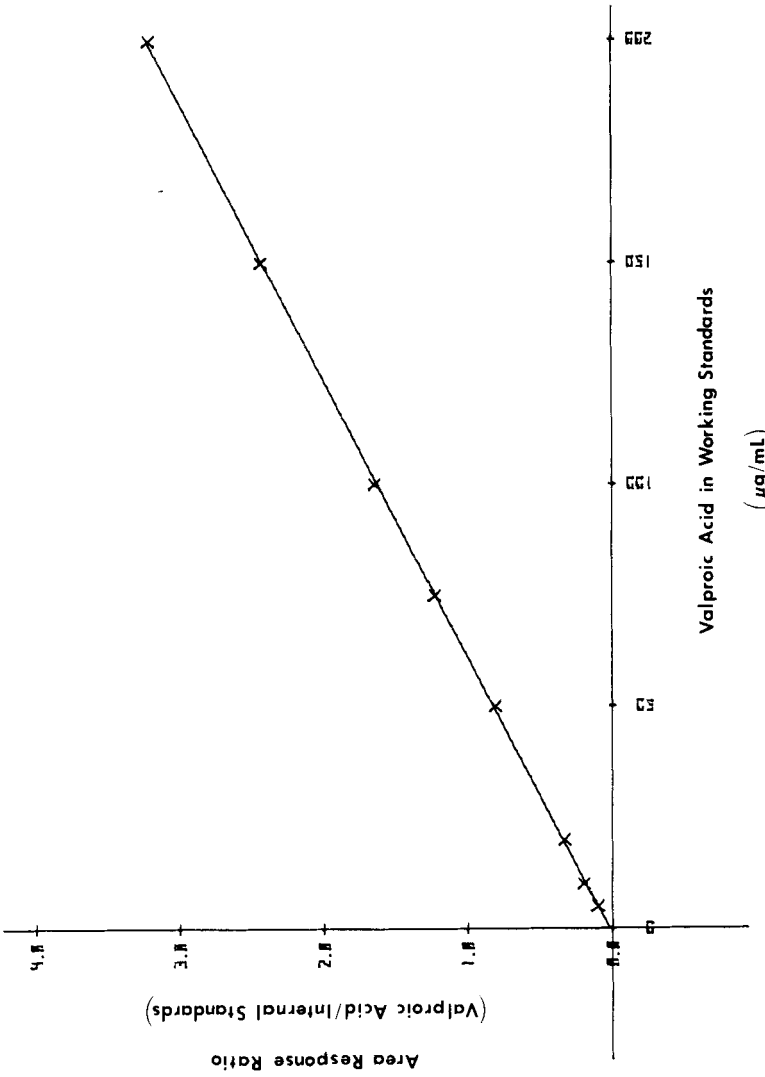

FIGURE 3. Valproic acid calibration curve. Response ratio is the detector response for valproic to that of cyclohexane carboxylic acid added at the 50 (μg/g) level as an internal standard.

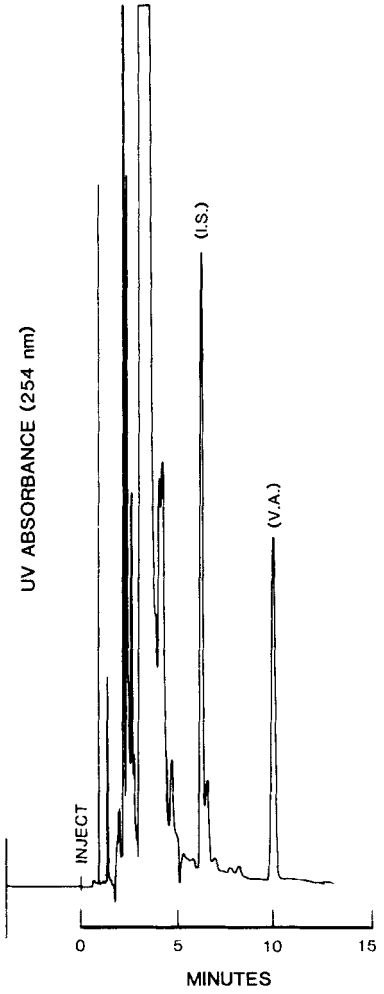

FIGURE 4. HPLC chromatogram of a patient serum sample. I.S. = cyclohexane carboxylic acid, V.A. = valproic acid.

The results obtained from the HPLC analysis of several patient serum samples are compared to the original clinical analysis supplied by Dudley in table 2. Figure 4 is a chromatogram of one of the patient serum samples.

TABLE 2

HPLC Analysis of Patient Serum Samples

	Valproic Acid Concentration (μg/mL)	
Sample No.	NBS-HPLC	NC-GC
1	85 ± 2[a]	79
2	40 ± 2	37
3	56 ± 2	67
4	67 ± 2	69
5	54 ± 3	58

[a]Uncertainties reported represent one standard deviation (n = 6).

SUMMARY

The procedure that we have described was developed for the analysis of valproic acid in a processed freeze dried human serum sample that is being issued as NBS SRM 1599. We feel, however, that this method has the simplicity, rapidity, accuracy, and precision necessary to be used routinely in the clinical laboratory.

A clinical laboratory faced with the analysis of large numbers of samples would view the procedure used for analysis of SRM 1599 as too time-consuming. We found three key areas where we could substantially reduce the analysis time, thereby making the method sufficiently rapid for a clinical laboratory. First, we found that the reaction incubation time could be reduced from 2 h to as short as 30 min. Secondly we found that, we could reduce the amount of derivatizing reagent from 0.5 to 0.1 mL, thereby eliminating a number of large non-analyte peaks from the chromatogram. This allowed us to use isocratic conditions and thereby reduce the HPLC run time from 40 min to 10 min (see figures 2 vs. 4.).

The data given in tables 1 and 2 show this method to yield results comparable to those of the GC methods commonly used for this assay. Therefore we feel that this method is a valuable additional tool that can be confidently used by laboratories faced with the problem of analyzing valproic acid in biological samples. In addition, this method is also applicable to the analyses of other carboxylic acids. For example, we have recently applied this procedure to the analysis of butyric, pentanoic and hexanoic acid in wastewater samples.

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