Journal of Chromatography, 432 (1988) 185–197
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4367

CAPILLARY GAS CHROMATOGRAPHIC ASSAY FOR VALPROIC ACID AND ITS 2-DESATURATED METABOLITE IN BRAIN AND PLASMA

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(First received March 15th, 1988; revised manuscript received June 28th, 1988)

SUMMARY

A capillary gas chromatographic method has been developed for quantitating the antiepileptic drug valproic acid and its pharmacologically active metabolite, E-Δ^2-valproic acid, in brain and plasma. The method was designed to cover large concentration ranges of both valproic acid and E-Δ^2-valproic acid for pharmacokinetic and pharmacodynamic studies in laboratory animals and human subjects. Careful optimization of the extraction and chromatographic procedures was needed to resolve the analytes from a variety of endogenous constituents and other known metabolites of valproic acid. A sensitivity limit of 0.10 μg/g for 300 mg of brain tissue or 0.20 μg/ml for 150 μl of plasma was achieved using flame ionization detection. The within-batch coefficients of variation for both analytes were less than 8%. Reproducible calibration data were observed over a period of three to eleven months.

INTRODUCTION

Valproic acid (2-*n*-propylpentanoic acid, VPA) is an anticonvulsant drug currently used to treat various types of generalized epilepsies, particularly absence and generalized tonic–clonic seizures. Recent clinical studies indicated that it is also effective as an adjunct agent in the treatment of partial seizures [1]. This broad spectrum of anticonvulsant activity may be associated with its unique branched-chain fatty acid structure [2]. Despite its efficacy, the use of valproic acid is curtailed in young children and pregnant women due to concern over VPA's potential to induce hepatotoxicity and fetal toxicity [3,4].

Studies in rodent seizure models have indicated that several of the unsaturated metabolites of valproic acid exhibit significant anticonvulsant activity [5–7]. The major circulating mono-unsaturated metabolite of VPA is the E-isomer of Δ^2-valproic acid (2-*n*-propyl-2-pentenoic acid, E-Δ^2-VPA) [8–10]. E-Δ^2-VPA attracted considerable attention when recent animal studies showed that it does not induce hepatic damage or significant embryotoxicity [5,11,12]. Therefore, E-

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Δ^2-VPA could potentially be offered as a safe and efficacious alternative to VPA. Animal studies focused on the pharmacokinetics and pharmacodynamics of *E*-Δ^2-VPA are presently being conducted in our laboratory.

The purpose of this report is to present a sensitive, capillary gas chromatographic (GC) assay using flame ionization detection (FID) for the measurement of VPA and *E*-Δ^2-VPA in brain tissue and plasma. Löscher [8] and Schäfer and Luhrs [13] have described packed column GC–FID assays for the unsaturated metabolites of VPA in plasma. The sensitivity of these earlier procedures was reported to be about 1–2 μg/ml for 0.5 ml of plasma. Our studies required assay sensitivity in the submicrogram per milliliter range with sample volumes less than 0.1–0.2 ml. Specificity is another concern, since many of the geometric isomers and newly discovered di-unsaturated metabolites of VPA cannot be adequately resolved on packed columns [14,15]. Methods utilizing capillary GC–mass spectrometry (MS) have recently been developed for valproate metabolism studies [9,15–17]. Although these MS procedures are highly sensitive (ca. 0.01–0.1 μg/ml in less than 1 ml of specimen) and specific, they require regular access to GC–MS instrumentation. Therefore, we decided to develop an alternative procedure using conventional capillary GC that will meet the demands of high throughput for pharmacokinetic and pharmacodynamic studies with VPA and *E*-Δ^2-VPA. Through careful optimization of our capillary GC–FID system, we were able to achieve a sensitivity limit of approximately 0.1–0.2 μg/ml in 150 μl of plasma or 300 mg of brain tissue, approaching that reported for GC–MS methods.

EXPERIMENTAL

Materials and reagents

VPA was purchased from Saber Labs. (Mortongrove, IL, U.S.A.) and used without further purification. Synthetic metabolites of VPA [2-*n*-propyl-4-pentenoic acid (Δ^4-VPA), 2-*n*-propyl-3-pentenoic acid (Δ^3-VPA), *E*- and *Z*-isomers of 2-*n*-propyl-2-pentenoic acid (*E*-Δ^2-VPA and *Z*-Δ^2-VPA), 2-(1′-propenyl)-2-pentenoic acid (*E,E*-$\Delta^{2,3'}$-VPA and *Z,E*-$\Delta^{2,3'}$-VPA) and 2-*n*-propyl-2,4-pentadienoic acid (*E*-$\Delta^{2,4}$-VPA, *Z*-$\Delta^{2,4}$-VPA)] were kindly provided by Dr. Thomas A. Baillie (Department of Medicinal Chemistry, University of Washington, Seattle, WA, U.S.A.). The internal standard, cyclohexane carboxylic acid (CHCA), was obtained from Eastman Organic Chemicals (Rochester, NY, U.S.A.). High-purity chloroform (preserved with amylene) and methanol were purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). The derivatizing reagent, bis(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Supelco (Bellefonte, PA, U.S.A.). All other reagent-grade chemicals, including magnesium sulfate anhydrous powder, hydrochloric acid and sodium hydroxide, were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.).

Preparation of plasma and brain standards

Concentrated stock solutions of VPA and *E*-Δ^2-VPA were prepared separately in methanol. Working solutions were prepared by serial dilution of the stock solutions with methanol and stored at 4 °C. Ten working solutions were prepared,

ranging from 0.0711 μg per 20 μl to 36.42 μg per 20 μl for VPA and from 0.0402 μg per 20 μl to 20.60 μg per 20 μl for E-Δ^2-VPA (see Table I). The internal standard (CHCA) solution was prepared in methanol at a concentration of 2.112 μg per 100 μl and stored at 4 °C. VPA, E-Δ^2-VPA and CHCA were stable in methanol up to eleven months as judged by the length of time over which reproducible calibration curves can be obtained for a given batch of working solutions.

On the day of analysis, standards were prepared by spiking blank plasma or brain tissue homogenate with 20 μl of the working solutions and 100 μl of internal standard solution. Spiked calibration standards plus a blank sample were prepared along with every batch of samples.

Extraction procedure for brain tissue

Brain tissue was obtained from male Sprague–Dawley rats. The whole brain was removed from the skull immediately after decapitation, rinsed thoroughly with normal saline to remove adhered blood and stored at −70 °C. On the day of sample preparation, the frozen whole brain was sectioned sagitally into halves (500–800 mg per half brain) and weighed after defrosting. One part of tissue was combined with two volumes of distilled, deionized water (1:2, w/v) in a hand-held Potter-Elvehjem tissue grinder and homogenized to a uniform consistency. A 100- to 900-μl aliquot of this homogenate, containing 33.3 to 300 mg of brain tissue, was transferred with a positive displacement pipet into an 8-ml glass culture tube for further processing. Fig. 1 outlines the extraction procedure subsequent to tissue homogenization. Internal standard was added to the homogenate after transfer to the culture tube. The homogenate was acidified with 5 M hydro-

TABLE I

ANALYTE CONCENTRATIONS IN WORKING SOLUTIONS AND BRAIN HOMOGENATE OR PLASMA STANDARDS

Analyte refers to either VPA or E-Δ^2-VPA.

Working solutions (μg per 20 μl)	Brain standards* (μg/g)	Plasma standards** (μg/ml)
Low calibration curve		
0.04024	0.1341	0.2683
0.08048	0.2683	0.5365
0.1610	0.5367	1.073
0.3218	1.073	2.145
0.6438	2.145	4.292
High calibration curve		
1.288	4.293	8.587
2.575	8.587	17.17
5.150	17.17	34.33
10.30	34.33	68.67
20.60	68.67	137.3

*Sample size, 300 mg of brain tissue in 900 μl of homogenate.

**Sample size, 150 μl.

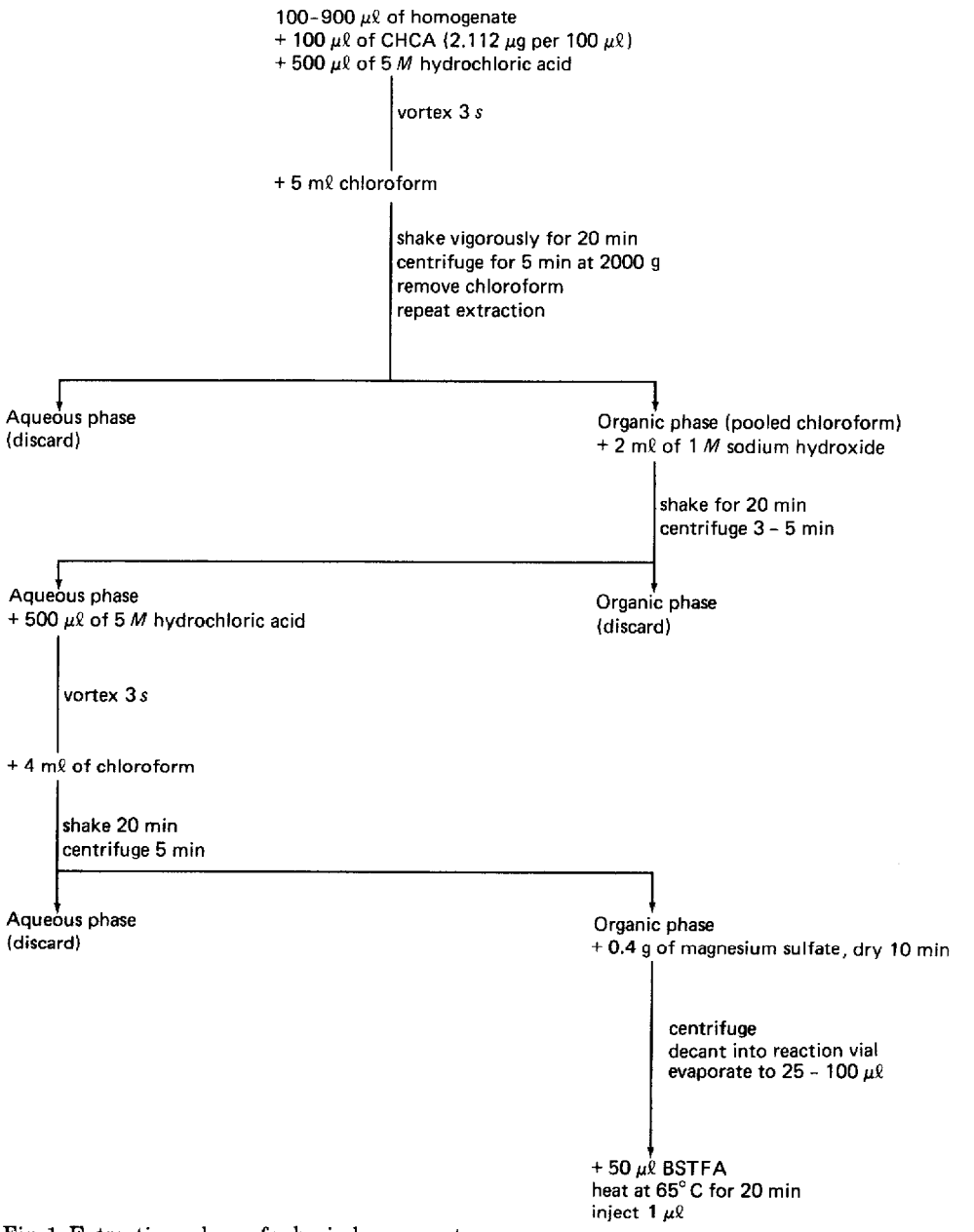

Fig. 1. Extraction scheme for brain homogenates.

chloric acid to precipitate proteins and provide an optimal pH for extraction of VPA and E-Δ^2-VPA ($pK_a = 5.0$). The acidified homogenate was extracted twice with 5 ml of chloroform.

Sample clean-up was accomplished by a back-extraction from chloroform into 2 ml of 1 M sodium hydroxide, which removed neutral endogenous substances that caused significant chromatographic interference. The back-extract was then

acidified with 500 μl of 5 *M* hydrochloric acid and extracted once with 4 ml of chloroform. This final chloroform phase containing VPA and *E*-Δ^2-VPA was dried over anhydrous magnesium sulfate powder for 10 min, decanted into a 5-ml reaction vial (Alltech Assoc., Deerfield, IL, U.S.A.) and evaporated to a small volume, 25–100 μl, under dry nitrogen. Care was taken to avoid evaporation to complete dryness, which results in significant loss of analyte and internal standard due to volatilization. Excess BSTFA (50 μl, neat) was added to the reaction vial and allowed to react for 20 min at 65 °C. A 1-μl volume of this solution was injected into the gas chromatograph. The trimethylsilyl (TMS) ester derivatives of VPA and *E*-Δ^2-VPA in these extracts were stable for at least 96 h at room temperature.

Extraction procedure for plasma or serum

Fig. 2 outlines the extraction procedure for plasma or serum samples. The anticoagulant for plasma was disodium ethylenediaminetetraacetate (EDTA). Aside from a minor adjustment in organic solvent/aqueous phase volume ratio and deletion of the back-extraction step, the handling of plasma/serum specimen is similar to that of tissue homogenate.

Capillary gas chromatography

A Hewlett-Packard Model 5890 gas chromatograph equipped with a split/splitless capillary inlet and a flame ionization detector was used. Samples were injected with a Hewlett-Packard Model 7673A autosampler and detector signal was processed by a Hewlett-Packard Model 3390A integrator.

Separation was achieved on a 60 m × 0.25 mm I.D. fused-silica capillary column

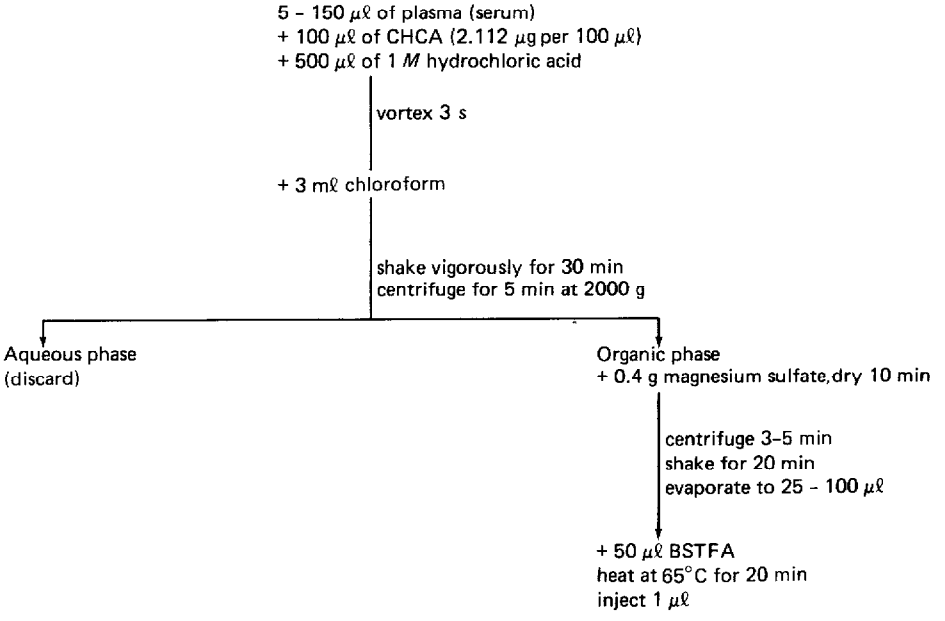

Fig. 2. Extraction scheme for plasma.

(J&W Scientific, Folsom, CA, U.S.A.), with a 0.25 μm thick cross-linked and chemically bonded dimethylpolysiloxane stationary phase (DB-1). The injector was optimized for low sample load in the "splitless" mode. The inlet insert was purged 60 s after injection. The injector split vent flow-rate was set at 100 ml/min, purge vent flow-rate was 2.5 ml/min and injector temperature was 200°C. At an oven temperature of 180°C and a column head pressure of 165 kPa, the linear velocity of hydrogen carrier gas was 40 cm/s. The carrier gas line had several gas purifiers in series, starting with molecular sieve/drierite (Alltech Assoc.), a charcoal gas purifier (Alltech Assoc.), an oxygen scrubber (Supelco, Bellefonte, PA, U.S.A.) and finally an oxygen indicator (Alltech Assoc.). The flame ionization detector temperature was set at 270°C.

The optimal air and hydrogen flow-rates for maximum detection sensitivity were 270 and 33 ml/min, respectively. Nitrogen was chosen as the detector make-up gas at a flow-rate of 38 ml/min.

After injection, the initial oven temperature at 40°C was held for 0.5 min, to allow cold trapping, followed by a rapid increase at 40°C/min to 80°C and held there for 2 min, then a slower ramp of 10°C/min to 250°C. The final temperature was held for 3 min in the case of plasma extracts and 10 min in the case of brain homogenate extracts. Over 50 plasma or 36 brain samples can be analyzed in 24 h.

Calibration

At least five different plasma or brain tissue standards covering the expected sample concentration range were processed together with each batch of samples. The calibration curve was established by linear least-squares regression of the peak-area ratios of analyte to internal standard against the concentration or amount of analyte. The regression parameters were used to calculate the amount of drug in samples from the measured peak-area ratios.

Extraction recovery

Blank brain homogenate or plasma was spiked with a known amount of analyte and processed as described in the extraction procedure section, but without the addition of CHCA at the start. The chloroform extracts were evaporated to a volume of approximately 50 μl. A known amount of CHCA in 50 μl of dry chloroform plus 50 μl of BSTFA were added after evaporation. The addition of CHCA as an external standard corrects for variation in the final volume of chloroform extract after evaporation. The reaction mixture was heated for 20 min at 65°C and injected on column. Six replicates were prepared this way and the mean peak-area ratio of analyte to CHCA was calculated (ratio A). In a separate set of reaction vials, the same amount of analyte and CHCA in 50 μl of dry chloroform were reacted with BSTFA and injected on column. The peak-area ratio of analyte to CHCA was calculated from this direct derivatization (ratio B). Ratio A divided by ratio B provided an estimate of total recovery of analytes.

Evaluation of intra- and inter-day reproducibility

The intra-day or within-batch reproducibility was assessed by replicate analysis ($n=6$) of spiked brain homogenate, plasma and serum over a wide concentration range.

Two parameters were used to evaluate inter-day reproducibility in the calibration procedure: peak-area ratio for a given calibrator and regression slope of the calibration curve. Calibration data gathered over a period of three to eleven months were compiled. The same analyte and internal standard working solutions were used over this period. For a given calibration standard, the coefficient of variation in peak-area ratios of analyte/internal standard from all assay runs were estimated. The between-run coefficient of variation in the regression slope of the calibration curves was also calculated.

RESULTS AND DISCUSSION

The advantages of the present GC procedure are its sensitivity and specificity as well as its adaptability to automation. The improvements in sensitivity and specificity over the earlier conventional GC procedures was achieved by careful refinements in sample preparation and chromatography.

Sample preparation

Analysis of valproate metabolites in brain tissue has previously been described by Nau et al. [16] using GC–MS methodology. Our initial attempt to adopt their tissue extraction procedure failed due to poor recovery and the appearance of large, late eluting peaks on the chromatograms. The interfering peaks most likely were derived from volatile endogenous constituents such as medium- and long-chain fatty acids and keto acids. Therefore, an improved sample clean-up procedure was developed for brain tissue.

Ethyl acetate was used in the solvent extraction of brain homogenates by Nau et al. [16]. The sample was buffered to a relatively high pH of 5.0, which may account for our experience of poor recovery. The extent and consistency in recovery improved by lowering the extraction pH to around 2 (below the pK_a of the analytes) and extracting twice with ethyl acetate. However, at the low pH large late eluting peaks appeared. These late eluates tended to build up on column during repeated injections of brain homogenate samples, eventually causing significant interference problems. Extraction with other solvents such as chloroform, hexane, heptane and diethyl ether did not eliminate the interfering materials. The problem was solved by incorporating a back-extraction step, which dramatically improved the chromatography. It should be noted that chloroform was selected as a substitute solvent, since the addition of a back-extraction step led to recovery problems when ethyl acetate was used as the extracting solvent.

Several commercially available silylating reagents were investigated with respect to background interference. N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA, Pierce, Rockford, IL, U.S.A.), N,O-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane (BSTFA+1% TMCS, Pierce) and

N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA, Pierce) all contained contaminants which co-chromatographed with the VPA and *E*-Δ^2-VPA derivatives. The cleanest reagent was pure BSTFA (Supelco). The BSTFA was added directly to the extract concentrated by careful evaporation in a conical reaction vial. The derivatization of VPA and *E*-Δ^2-VPA was complete after heating the extract reagent solution for 20 min at 65 °C.

Chromatographic optimization

A 60-m thin-film DB-1 capillary column provided the best separation of the TMS ester derivatives of VPA and the mono-unsaturated metabolites. The cross-linked, chemically bonded stationary phase of methylsilicone was very stable and maintained its performance after several thousand injections. Fig. 3 shows a chromatogram of brain tissue extract spiked with VPA, the mono-unsaturated metabolites, two of the major di-unsaturated metabolites and the internal standard, CHCA. The retention times of these compounds are listed in Table II. Fig. 4A

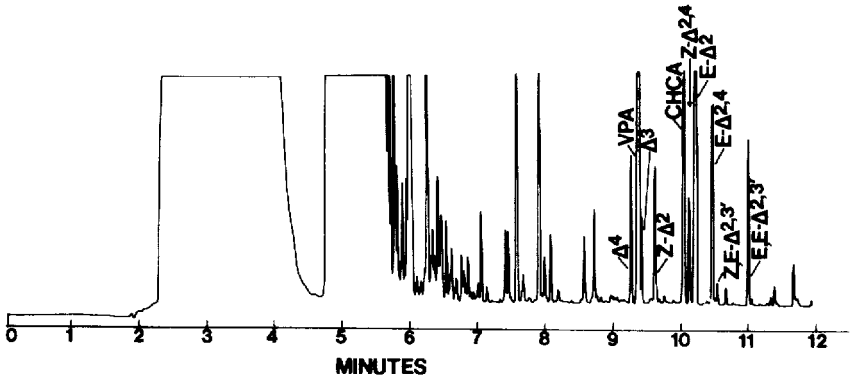

Fig. 3. Chromatogram of blank rat brain homogenate spiked with all of the compounds listed in Table II.

TABLE II

RETENTION TIMES OF VPA AND THE UNSATURATED DERIVATIVES

Compound	Retention time (min)
Δ^4-VPA	9.29
VPA	9.39
Δ^3-VPA	9.44
Z-Δ^2-VPA	9.64
CHCA	10.07
Z-$\Delta^{2,4}$-VPA	10.16
E-Δ^2-VPA	10.24
E-$\Delta^{2,4}$-VPA	10.49
Z,E-$\Delta^{2,3'}$-VPA	10.58
E,E-$\Delta^{2,3'}$-VPA	11.02

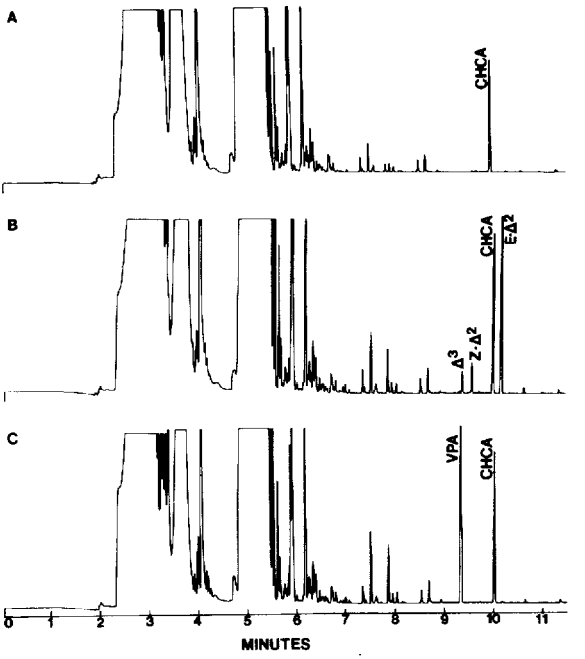

Fig. 4. Chromatograms of rat brain extracts. (A) Blank brain homogenate spiked with internal standard. (B and C) Actual brain samples obtained 15 min after an intravenous dose of E-Δ^2-VPA and VPA, respectively. The assayed concentrations were 24.29 μg/g for E-Δ^2-VPA and 29.76 μg/g for VPA.

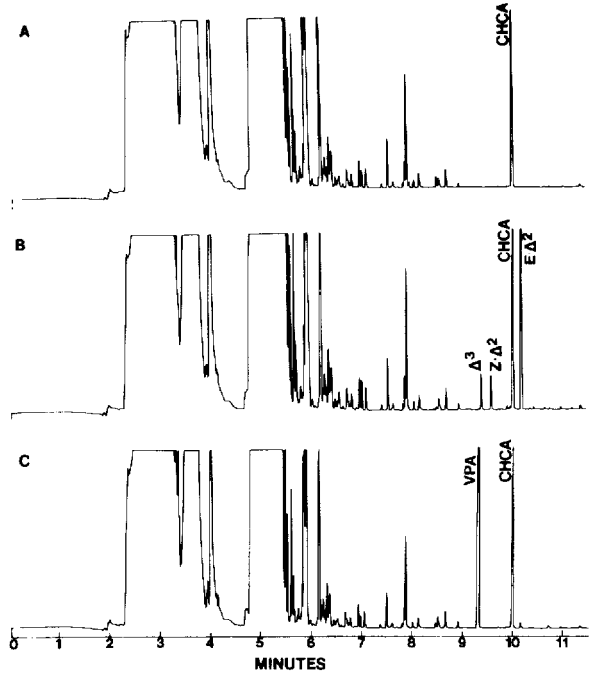

Fig. 5. Chromatograms of rat plasma extracts. (A) Blank rat plasma spiked with internal standard. (B and C) Actual rat plasma samples obtained 15 min after an intravenous dose of E-Δ^2-VPA and VPA, respectively. The assayed concentrations were 112.9 μg/ml for E-Δ^2-VPA and 180.1 μg/ml for VPA.

shows a chromatogram of rat brain tissue extract spiked with internal standard, demonstrating the absence of interfering endogenous peaks. Fig. 4B and C are chromatograms of actual brain extracts obtained from in vivo studies after administration of E-Δ^2-VPA and VPA, respectively. Fig. 5 shows a parallel set of chromatograms for rat plasma extracts.

Except for Δ^3-VPA and VPA, all of the analytes were well resolved from each other. Δ^3-VPA overlapped slightly with VPA (resolution factor ~ 1.0). This does not pose a problem with the quantitation of VPA since Δ^3-VPA is a minor metabolite, usually present in brain or plasma at concentrations less than 1% that of the parent compound. Improved resolution between VPA and Δ^3-VPA can be achieved with the more polar DB-5 stationary phase. However, incomplete resolution between VPA and Δ^4-VPA occurred with the latter column.

TMS derivatives of other known oxidative metabolites, such as 3-keto-VPA, 3-OH-VPA, 4-OH-VPA, 5-OH-VPA and 2-propylglutaric acid, did not coelute with analytes or CHCA. In addition, no chromatographic interference by endogenous substances in brain tissue or plasma with any of the unsaturated metabolites or internal standard has been observed during our experience with hundreds of specimens from rats and human subjects.

Detector optimization

Originally, helium was employed as make-up and carrier gas. Switching the make-up gas to nitrogen improved detector sensitivity by two-fold. When helium carrier gas was replaced with hydrogen, an additional three-fold increase in sensitivity was noted.

The most important step in the temperature programming was the initial cold trapping at 40 °C. This step was essential to achieve sharp peak shapes and optimal detection sensitivity. The second temperature ramp from 80 to 250 °C was adjusted to maximize peak separation and minimize chromatographic run time.

Linearity, sensitivity and specificity

Linear least-squares regression was used to fit the calibration curves. Separate calibration plots were constructed for low and high concentration ranges depending on the expected concentration of the specimen. The low and high calibrator concentrations are listed in Table I. Limiting the calibration concentration range insured accurate interpolation and avoided least-squares weighting problems.

The limit of detection for on column injection was 100 pg, with a signal-to-noise ratio of 10:1. The sensitivity limit for actual sample was 0.10 μg/g for 300 mg of brain and 0.20 μg/ml for 150 μl of plasma (see Table III).

Peak purity of VPA and E-Δ^2-VPA was verified by MS as described by Rettenmeier and co-workers [15,18]. The chromatographic and mass fragmentation characteristics of authentic standards of VPA and E-Δ^2-VPA were analyzed to determine the precise retention time and the ratios of ion current intensities of the predominant characteristic ions. Rat plasma samples obtained after a single intravenous dose of VPA or E-Δ^2-VPA were extracted and analyzed under identical conditions. Mass spectra were collected on succeeding fractions of the eluting analyte peak. The peak was judged to be pure based on retention time

TABLE III

PERFORMANCE MEASURES AND RECOVERY ESTIMATES FOR THE ASSAY OF VPA AND E-Δ^2-VPA IN BRAIN, PLASMA AND SERUM

Sample size of 300 mg for brain and 150 μl for plasma or serum.

Sample	Analyte	Mean concentration (μg/g or μg/ml)		Intra-batch coefficient of variation ($n=6$) (%)	Recovery (mean ± S.D., $n=6$) (%)
		Expected	Measured		
Brain	E-Δ^2-VPA	0.134	0.133	6.57	73.0 ± 5.18
		4.29	4.31	1.01	60.4 ± 9.36
	VPA	0.237	0.262	6.67	85.5 ± 5.29
		7.59	7.81	2.72	63.6 ± 10.6
Plasma	E-Δ^2-VPA	0.268	0.288	7.55	69.7 ± 5.22
		2.15	2.04	2.89	55.8 ± 5.28
		34.3	33.9	5.11	57.8 ± 5.05
	VPA	0.474	0.437	4.38	81.6 ± 7.51
		3.79	3.79	3.92	58.3 ± 4.95
		121.4	121.6	2.62	61.2 ± 4.17
Serum	E-Δ^2-VPA	0.268	0.303	6.33	66.1 ± 4.72
		2.15	2.03	1.09	63.5 ± 2.65
	VPA	0.474	0.499	1.49	90.0 ± 6.28
		3.79	3.74	0.83	68.4 ± 3.39

comparison with the authentic standards and the ratios of ion current intensities of characteristic ions in each peak fraction.

Assay performance

Intra-batch reproducibility of the assay was assessed by replicate analysis of spiked brain and plasma samples (Table III). For brain homogenate, reproducibility was checked at low (<1 μg/g) and intermediate (1–10 μg/g) concentrations. For plasma/serum, assessment was performed at three levels of analyte concentration: low (<1 μg/ml), intermediate (1–10 μg/ml) and high (10–150 μg/ml) concentrations.

The within-batch coefficient of variation for both VPA and E-Δ^2-VPA ranged from 0.83 to 7.55% (see Table III). Recovery from brain tissue homogenate ranged from 60 to 86%, while plasma recovery ranged from 56 to 82% and serum recovery from 65 to 90%. Published recovery data for VPA and metabolites using ethyl acetate as the extracting solvent ranged from 75 to 97% [9,16]. Recovery was consistently higher at the low concentration (<1 μg/ml) in both plasma and brain homogenate. The effect of this apparent concentration-dependent recovery on the linearity of the calibration curves was minimized by dividing the 500-fold range standards into low and high calibration sets as shown in Table I. Limiting the calibration range was also desirable in order to avoid weighting problems in the linear least-squares regression analysis.

Table IV lists the parameters indicating the inter-day reproducibility of the calibration curves. The calibration curve stability assessment was obtained with

TABLE IV

INTER-DAY CALIBRATION CURVE STABILITY

Sample type	Analyte	Amount of analyte added (μg)	Number of calibration curves	Time interval (months)	Coefficient of variation (%)	
					Peak-area ratio*	Slope
Brain	E-Δ^2-VPA	0.6438	6	4	2.04	1.99
		10.30	6	4	1.86	
	VPA	1.138	5	3	6.98	2.45
		18.21	5	3	1.20	
Plasma	E-Δ^2-VPA	0.6438	26	11	6.71	3.47
		10.30	26	11	3.63	
	VPA	1.138	6	3	3.24	4.31
		18.21	6	3	2.08	

*Peak-area ratio of analyte/internal standard.

the high-concentration calibrators. In practice, this was the most frequently used set of standards. The inter-day coefficients of variation in peak-area ratios at two levels of the calibration curve were less than 7%, indicating a high degree of reproducibility in extraction and instrument response. The coefficients of variation of the slope parameter were all less than 5%.

Application of the method to other biological samples

The assay procedure initially developed for plasma and brain tissue from rats has since been successfully applied to a variety of biological specimens from other species, such as human plasma, mouse serum, plasma and cerebrospinal fluid from monkeys, urine, bile and whole blood from rats. The brain extraction procedure has also been applied to mouse and human brain samples with good results. Several thousand samples have been analyzed to date using this procedure.

ACKNOWLEDGEMENTS

We would like to thank Dr. Albert W. Rettenmeier for GC–MS validation of assay specificity. We are also indebted to Dr. Elizabeth Kwong for her valuable advice. This work was supported, in part, by a United States Public Health Services Grant NS-22662 and by the Arthur A. Denny Graduate Fellowship Award to R.L.O. Semmes from the Graduate School, University of Washington.

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