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Methods to reduce background interferences in electron-capture gas chromatographic analysis of valproic acid and its unsaturated metabolites after derivatization with pentafluorobenzyl bromide

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Abstract

Analysis of the branched, medium-chain fatty acid anticonvulsant, valproic acid, and its unsaturated metabolites by gas chromatography with electron-capture detection suffered from background interference caused by the derivatizing reagent pentafluorobenzyl bromide. Background was reduced by keeping the derivatization anhydrous, using an inert solvent, minimizing the amount of pentafluorobenzyl bromide, using hypernucleophilic bases and displacing the derivatization solvent with isooctane. However, these strategies proved difficult to reproduce. Post-derivatization clean-up with HPLC was much more reliable and provided sufficient sensitivity for the analysis of extracts of plasma and brain homogenate. The assay was validated for plasma and brain samples from humans, rats and mice.

Keywords: Valproic acid; Medium-chain fatty acids; Fatty acids; Pentafluorobenzyl bromide

1. Introduction

Pentafluorobenzyl bromide (PFB-Br) is a common reagent for converting carboxylic acid analytes to electrophilic derivatives, which allows sensitive quantitation by electron-capture detection (ECD) in gas chromatographic (GC) analysis [1–3]. Unfortunately, maximum sensitivity is often limited by derivatization-induced interference, or background [2]. We investigated methods to reduce background problems that were encountered during the development of a GC–ECD assay for the anticonvulsant alkanoate, valproic acid (2-*n*-propylpentanoic acid; VPA), and its monoene and diene metabolites employing PFB derivatization.

Several of the unsaturated metabolites of VPA, namely 2-*n*-propyl-*E*-2-pentenoic acid (Δ^2-VPA), 2-(*E*-1-propenyl)-*E*-2-pentenoic acid ($\Delta^{2,3'}$-VPA) and 2-*n*-propyl-3-pentenoic acid (Δ^3-VPA) possess anticonvulsant activity in animal models of seizures [4]. Another metabolite, 2-*n*-propyl-4-pentenoic acid (Δ^4-VPA), is hepatotoxic [5] and teratogenic [6]. Typical anticonvulsant levels for VPA are 50–100 μg/ml in plasma [7–9] and concentrations of unsaturated metabolites are 1–10% of those of the parent drug [4,8,9]. Brain-to-plasma concentration ratios of these metabolites range from 0.1–0.01 [7,8,10]. Brain VPA and metabolite concentrations that are much less than 1 μg/g are achieved during

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pharmacological studies. A sensitive assay was needed to quantify these low drug metabolite concentrations in small samples (50 mg) of brain tissue that were obtained from human surgical resections and from studies using rodents.

Numerous methods for the clinical monitoring of VPA in plasma have been published [11,12]. More sensitive GC–MS methods have been developed for the trimethylsilyl, *tert.*-butyldimethylsilyl or methyl esters of VPA and its unsaturated metabolites in plasma, using packed [10,13,14] or capillary [9,15–17] columns. Only capillary columns were able to resolve the multiple dienes and monoene metabolites [15–17]. Further sensitivity was gained through quantitation of the PFB esters of VPA and its metabolites by negative-ion chemical ionization GC–MS [18]. One study has quantitated VPA in serum using GC–ECD after derivatization with phenacyl bromide [19].

Few tissue assays are available for VPA and its unsaturated metabolites. VPA and Δ^2-VPA were quantitated in brain homogenates by flame ionization detection (FID) in a capillary GC assay [20] and by packed column GC–MS [10]. VPA, Δ^2-VPA and Δ^4-VPA were measured in several tissues by packed column GC–MS [14]. A sensitive assay to simultaneously measure valproic acid and all of its major unsaturated metabolites in brain has not been reported.

First demonstrated by Kawahara [21], PFB-Br reacts readily with carboxylic acids to form ECD-sensitive esters with good gas chromatographic properties. Johnson [22] noted that Kawahara's methodology did not work well at nanogram levels due to loss through sample work-up, particularly for volatile esters. Since then, many modifications of the derivatization and clean-up have been tried, to reduce excessive background and loss of analyte [23,24]. The modifications can be grouped by method of alkylation: liquid–liquid extractive reaction [24–27], solid–liquid phase-transfer catalysis [28,29], organic base catalysis [30] and impregnated resin catalysis [31]. Of the methods reviewed, the most sensitive and free from background was extractive alkylation, which achieved quantitation at ng levels for long chain *n*-alkyl carboxylic acids [27]. However, this method would not be easily adapted to tissue samples because of emulsion formation. Sensitivity in assays using alkylation by phase-transfer catalysis were limited by derivatization-induced background. Resin-catalyzed alkylation did not appear to offer any advantages in either lower background or higher sensitivity.

We present two methods for analysis of plasma and one for brain. For one of the plasma assays, conditions were designed to reduce the formation of background. Although sensitivity was still limited by background, the method was illustrative of factors contributing to background and of approaches to reduce them. The second plasma assay used HPLC for post-derivatization clean-up. It was much more robust and, unlike the first method, was easily adapted to tissue.

2. Experimental

2.1. Chemicals

Pentafluorobenzyl bromide (PFB-Br) was purchased from Pierce (Rockford, IL, USA). PFB-Br from this source had the least amount of impurities. PFB-Br was stored under dry nitrogen to prevent decomposition. 3-Ethyl-2-hexenoic acid (3ET) was obtained from the ABC Division of Aldrich (Milwaukee, WI, USA). Cyclohexane carboxylic acid (CHCA) was bought from Eastman (Rochester, NY, USA). Other alkyl carboxylic acids were obtained from Alltech Associates (Deerfield, IL, USA).

Valproic acid (2-*n*-propylpentanoic acid) was obtained from Saber Laboratories (Lot 8500601; Morton Grove, IL, USA). Analysis of this lot by thin-layer chromatography (TLC), GC–FID, GC–MS and GC–ECD on several types of columns showed it to be free of unsaturated VPA analogs. A previous lot contained Δ^3-VPA and Δ^2-VPA, as does pharmaceutical-grade VPA. 4,5-[^{3}H]-Valproic acid (868.5 mCi/mmol) was obtained through custom synthesis (New England Nuclear, Boston, MA). *E*-2-*n*-Propyl-2-pentenoic acid (*E*-Δ^2-VPA) was synthesized as described previously [15]. As used, it contained 4.1% *Z*-2-*n*-propyl-2-pentenoic acid (*Z*-Δ^2-VPA) and 2.6% 2-*n*-propyl-*E*-3-pentenoic acid (*E*-Δ^3-VPA). *E*-Δ^3-VPA, ethyl 2-(2-*n*-propenyl)-4-pentenoate (precursor to $\Delta^{4,4'}$-VPA), ethyl 2-*n*-propyl-*E*-2,4-penta-

dienoate (precursor to E-$\Delta^{2,4}$-VPA) and 2-*n*-propyl-4-pentenoic acid (Δ^4-VPA) were kindly supplied by Dr. Thomas A. Baillie (Department of Medicinal Chemistry, University of Washington). The ethyl esters were converted to free acids by hydrolysis over 16 h in 1 *M* methanolic sodium hydroxide. E-$\Delta^{2,4}$-VPA contained 27.1% of the *Z* isomer. 2-(1-*E*-Propenyl)-2-*E*-pentenoic acid (E,E-$\Delta^{2,3'}$-VPA) was synthesized [15] and contained 11.1% E,Z-$\Delta^{2,3'}$-VPA. Corrections were made for isomeric impurities in the preparation of calibration standards for each metabolite.

Other chemicals were purchased from Aldrich. Solvents were distilled-in-glass (Burdick and Jackson, Muskegon, WI, USA). Chloroform was preserved with amylene. Unpreserved tetrahydrofuran (THF) was distilled over sodium benzophenone immediately prior to use. Preservatives in THF produced massive interferences in the gas chromatograms. 4-N,N-Dimethylaminopyridine (DMAP) was purified by sublimation. DMAP and 4-pyrrolidinopyridine (PPY) were stored under dry nitrogen to retard oxidation. Their oxidation products also caused interferences in the gas chromatograms. Solutions of these compounds were prepared fresh prior to use.

2.2. Synthesis of PFB esters of VPA and its unsaturated metabolites

A modification of a previously described procedure was used [30]. Equimolar portions (2 mmol) of the carboxylic acid, PFB-Br and 1-ethylpiperidine were dissolved in 5 ml of acetone and heated at 60°C for 1 h in a sealed reaction vial. The mixture was added to 30 ml of water-saturated diethyl ether, quickly washed with 10 ml of 0.01 *M* NaOH and then by two washes with 10 ml of water. The ether layer was dried with magnesium sulfate and evaporated with a nitrogen stream. The residue, a yellow oil, was dissolved in 1 ml of hexane and applied to a 1-g silica column (made in a Pasteur pipette) [22]. The column had been prewashed with 10 ml of hexane. Four fractions were collected: (1 and 2) 8 ml of 5% benzene in hexane, (3) 6 ml of 25% benzene in hexane and (4) 8 ml of 75% benzene in hexane. Toluene should be an acceptable substitute for benzene. Fractions 3 and 4 contained the esters and were combined. Solvents were removed under reduced pressure, which left a pale yellow oil. The purity of the compounds was checked by TLC, GC–ECD, GC–MS and GC–FID. The absence of non-volatile impurities was confirmed by TLC (Silica-gel 60, F_{254}; EM Science, Cherry Hill, NJ, USA). Table 1 gives R_F values for VPA, its metabolites and their PFB esters.

2.3. Materials

Glassware was silanized with 5% dichlorodimethylsilane in petroleum ether and rinsed consecutively with methanol and dichloromethane. Siliconized micro-selecta-pipette tips (Clay Adams, Parsippany, NJ, USA) were used.

2.4. Animals and animal treatments

Carotid arterial blood was taken from rats. Trunk blood was taken from mice after decapitation. Ethylenediaminetetraacetic acid (EDTA) was present in the collection tubes. Blood was centrifuged for 10 min at 500 *g* to obtain plasma. Brain samples from the rodents were frozen in liquid nitrogen and stored at −70°C until analysis. Human brain samples were

Table 1
TLC of VPA, metabolites, recovery standards and PFB esters

Chemical	R_F of solvent system		
	A	B	C
VPA	0.59		0.09
VPA PFB ester	0.69	0.74	0.18
E-Δ^2-VPA	0.63		0.04
E-Δ^2-VPA PFB ester	0.70	0.63	0.15
Δ^3-VPA	0.63		0.02
Δ^3-VPA PFB ester	0.71	0.62	0.14
E-$\Delta^{2,4}$-VPA	0.62		0.07
E-$\Delta^{2,4}$-VPA PFB ester	0.70	0.63	0.15
E-Δ^3-VPA	0.67		
E-Δ^3-VPA PFB ester	0.68		0.05
E,E-$\Delta^{2,3'}$-VPA	0.61		0.01
E,E-$\Delta^{2,3'}$-VPA PFB ester	0.70	0.64	0.07
3ET	0.62		0.05
3ET PFB ester	0.70	0.62	0.15
CHCA	0.54		0.08
CHCA PFB ester	0.70	0.62	0.12

Solvent systems for TLC were (% by volume): (A) 97% ethyl acetate and 3% acetic acid; (B) 83% hexane, 8% acetone and 9% methanol; (C) 25% benzene and 75% hexane.

surgical waste from surgery performed on patients with epilepsy [32].

2.5. HPLC

The chromatographic system consisted of a Rheodyne 7120 injector (100-μl loop; Cotati, CA, USA), a Waters 6000A pump and a 440 UV detector (Millipore, Milford, MA, USA) set at 254 nm. A direct-connect guard column (20×2 mm I.D.; pellicular silica; Alltech Associates) was used with a Microsorb silica column (5 μm particle size; 250×4.6 mm I.D.; Rainin, Emeryville, CA, USA). The mobile phase for isocratic elution at 1.5 ml/min was prepared fresh daily; 7.5 ml of anhydrous diethyl ether and 280 μl of acetonitrile were combined and then brought up to 1 l with hexane. Strongly retained residues on the column were eluted with a consecutive treatment of at least 60 ml of THF, 60 ml of methanol and 60 ml of THF. This was necessary after every 20–25 sample injections, to maintain good chromatography. A mixture of VPA, E-Δ^{2}-VPA, hexanoic acid, E,E-$\Delta^{2,3'}$-VPA and cyclohexane carboxylic acid PFB esters (10 μl of a 10 μg/ml solution in isooctane) was routinely used to confirm the integrity of the HPLC separation.

2.6. Gas chromatography

The GC system was a Hewlett-Packard 5890A gas chromatograph (Avondale, PA, USA), with a 7673A autosampler and a 3390A integrator. A column (Stabilwax, 30 m×0.32 mm I.D., 0.25 μm film thickness; Restek, Bellefonte, PA, USA) with a Carbowax stationary phase completely resolved the unsaturated metabolites from VPA; columns with methyl, methyl–phenyl or cyano-phases did not resolve the metabolites. A fused-silica guard column (5 m×0.32 mm I.D., phenylmethylsiloxane, deactivated; Restek) was connected by a butt connector (Supelco, Bellefonte, PA, USA) to the analytical column.

Hydrogen carrier gas had a linear velocity of 45 cm/s at 150°C. Full resolution between some of the metabolites was not achieved with helium as the carrier gas. Splitless injections were made with injector purging after 0.5 min at a 60:1 purge ratio. The temperature program was 40°C for 0.5 min, 50°C/min to 120°C, 2°C/min to 150°C, then 50°C/min to 250°C, which was held for 5 min. The detector make-up gas was 10% methane in argon at a flow-rate of 50 ml/min. Optimum detector and injector temperatures were 280 and 200°C, respectively.

Oxygen and water traps installed on carrier and make-up gas lines reduced the ECD background signal. Thermogreen LB-2 septa (Supelco) for the injector gave the lowest background. Adsorption at the injection port contributed to peak tailing and loss, at low levels, of analytes. Therefore, a quartz splitless liner (2 mm I.D.; Hewlett-Packard) was silanized in place with an injection of a mixture of trimethylchlorosilane and hexamethyldisilazane at 300°C [33].

2.7. Sample preparation

2.7.1. Method 1. Analysis of plasma with reduction of ECD background through optimization of derivatization conditions

In a 1.5-ml polypropylene microcentrifuge tube, 50 μl of plasma was added to 0.5 ml of 1 *M* HCl, 0.8 ml of *n*-butylchloride and 50 μl of 1 μg/ml 3ET (recovery standard). This mixture was vortex-mixed for 1 min, centrifuged for 2 min in a microcentrifuge (Fisher Scientific Model 235B) and then frozen at −70°C. The organic layer was transferred to another microcentrifuge tube and evaporated with a stream of nitrogen to about 50 μl. The sealed tube was transferred to a glove bag, where subsequent operations were performed in a dry nitrogen atmosphere. THF (100 μl), 50 μl of a DMAP solution (42 mg/ml THF) and 50 μl of a PFB-Br solution (25 μl/2 ml THF) were added to the extract. After 1 h of reaction at room temperature, 1 ml of isooctane was added and the samples were taken out of the glove bag. The samples were then centrifuged for 1 min and the upper isooctane layer was analyzed directly on the GC. Quantitation was made with respect to the recovery standard.

2.7.2. Method 2. Analysis of plasma with reduction of ECD background through HPLC post-derivatization clean-up

Fig. 1 is a scheme for the clean-up of extracts from plasma and from brain homogenate. Brain

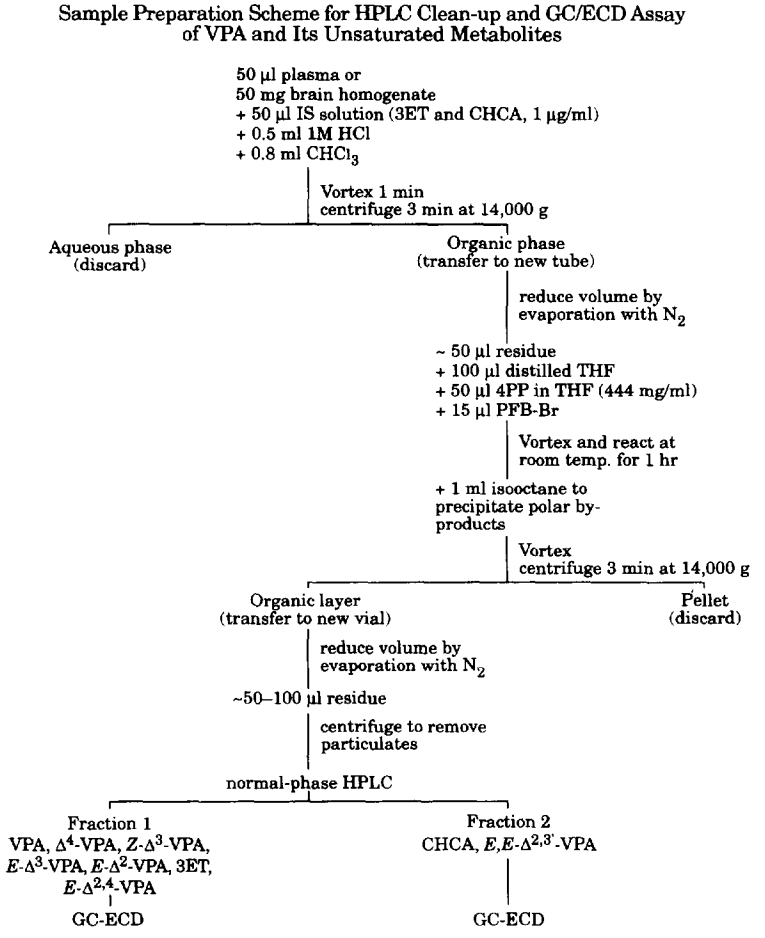

Fig. 1. Sample preparation scheme for GC–ECD assay with post-derivatization clean-up by HPLC for plasma or brain.

samples were as described in Section 2.7.3. In a 1.5-ml polypropylene microcentrifuge tube, 50 µl of plasma were added to 0.5 ml of 1 *M* HCl, 0.8 ml of chloroform and 50 µl of recovery standard solution (1 µg/ml 3ET and 1 µg/ml CHCA in chloroform). This mixture was vortex-mixed for 1 min and centrifuged for 3 min. The lower chloroform layer was transferred to another microcentrifuge tube and evaporated with a stream of nitrogen to about 50 µl. To the extract, 100 µl of THF, 50 µl of PPY solution (444 mg/ml THF) and 15 µl of PFB-Br were added. After 1 h, 1 ml of isooctane was added and the mixture was vortex-mixed. The samples were centrifuged for 2 min and the upper isooctane layer was transferred to another microcentrifuge tube. The isooctane solution was concentrated to approximately 50–100 µl with a stream of nitrogen and was injected onto the HPLC for fractionation. A large peak at about 3.6 min (Fig. 2), consisting of long-chain carboxylic acid PFB esters, served as a starting marker to collect the first fraction. Reproducible features of the liquid chromatogram around 5 min were markers for the division of the first and second fraction (Fig. 2). The first fraction contained PFB esters of 3ET, VPA and its unsaturated metabolites, except for *E,E*-$\Delta^{2,3'}$-VPA. Hexanoic acid, *E,E*-$\Delta^{2,3'}$-VPA and CHCA PFB esters eluted in the second fraction. Fractions were collected directly into a GC vial containing 16 ng of 2,3,4,5-tetramethylbromobenzene. This compound was an optional internal standard for calculation of absolute recoveries of analytes based on synthesized standards

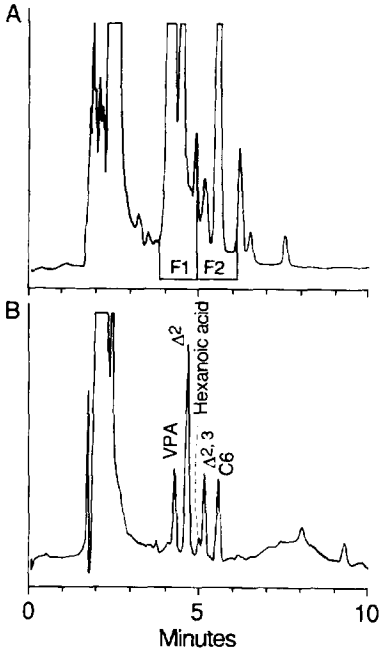

Fig. 2. HPLC was used to remove excess PFB-Br and other interferences from the derivatization mixture prior to GC. (A) Chromatogram of a 50-mg sample of human brain that was processed according to Method 3 (Section 2.7.3). F1 and F2 denote fractions containing analytes. (B) Chromatogram of selected PFB esters (10 μl of a 10-μg/ml solution in isooctane). This mixture of compounds was used to confirm the HPLC separation prior to clean-up of samples.

of PFB esters. The two fractions were then analyzed by GC–ECD. For increased sensitivity, the solvent volume in the vials can be reduced by a nitrogen stream.

With each set of samples, a sample blank and five calibration standards prepared by spiking blank plasma with analytes were processed and analyzed, to generate a standard curve. The blanks were either plasma or brain tissue from human subjects or animals that had not been exposed previously to VPA. Peak area ratios of analyte to recovery standard were plotted against spiked concentration. Slope and intercept of the standard curve were determined by weighted linear regression. The weighting factor was the reciprocal of concentration. CHCA was the recovery standard for E,E-$\Delta^{2,3'}$-VPA. The recovery standard for all other compounds was 3ET.

2.7.3. Method 3. Analysis of brain tissue with reduction of ECD background through HPLC post-derivatization clean-up

Brain tissue was analyzed using a slight modification of Method 2. A pestle was made (from a PTFE rod) to fit the bottom of the microfuge tube. A motor-driven polypropylene micro-homogenizer has become available (Pellet Pestle; Kontes, Vineland, NJ, USA). Small brain samples (50 mg) were homogenized in the microcentrifuge tube used for the extraction. To the brain tissue, 0.5 ml of 1 *M* HCl and 50 μl of the recovery standard mixture were added. This mixture was re-homogenized, followed by extraction with 0.8 ml of chloroform. The subsequent processing was as described in Section 2.7.2.

2.8. Method development and validation

Experiments were conducted to assess the recovery of analytes using the methods described above. A known amount (2.5 ng) of VPA, E-Δ^2-VPA and Δ^4-VPA were dissolved in 50 μl of THF. A 100-μl volume of THF, 50 μl of a DMAP solution (42 mg/ml THF) and 50 μl of PFB-Br solution (25 μl/2 ml of THF) were added to the solution of analytes and were reacted at room temperature in sealed reaction vials for 1 h. We identified peaks of the PFB derivatives in a gas chromatogram and calculated their absolute recoveries based on synthesized PFB esters. The effect of drying reagents on derivatization and recovery were also assessed.

For estimates of extraction efficiency, 2.5 ng of VPA with 0.05 μCi of [^{3}H]-PVA in 50 μl of methanol, spiked into blank plasma and brain homogenate, were used. Radioactivity (DPM) recovered in the extraction solvent was compared to an equal amount spiked into the same volume of extraction solvent. To assess evaporative losses during concentration, 50 μl of the radiolabeled VPA solution was added to an extraction solvent (0.8 ml). Various additives, the solvent and the method of solvent concentration were evaluated.

Recovery of PFB esters from the HPLC fractionation was evaluated by injection (50 μl) of standards of VPA, Δ^4-VPA, E-Δ^2-VPA, and E,E-$\Delta^{2,3'}$-VPA PFB esters. Two concentrations were evaluated for VPA, i.e., 0.1 and 10 μg/ml. Δ^4-VPA and E-Δ^2-VPA were evaluated at 0.1 μg/ml. Abso-

lute recoveries were calculated using internal standard and PFB ester calibrants.

Synthesis of the PFB esters allowed absolute quantitation of E-Δ^2-VPA, Δ^4-VPA, E,E-$\Delta^{2,3'}$-VPA, 3ET, CHCA and VPA. We had only sufficient amounts of Δ^3-VPA and E-$\Delta^{2,4}$-VPA to make PFB esters for rough quantitative comparisons. Response was defined as the ratio of analyte area to the area of the internal standard, 2,3,4,5-tetramethylbromobenzene, which was added to the sample immediately prior to injection onto the GC.

Absolute calibration curves for each PFB ester were constructed from 1-μl injections of standard solutions ranging from 1 pg to 100 ng on-column. Response curves were of the form: response$=a\times$ concentration^b+c. The equation was fit using weighted least-squares regression. The weighting factor was the reciprocal of concentration. Recoveries were based on stochiometric conversion of free acid to PFB ester. Because E-$\Delta^{2,4}$-VPA PFB ester was not available in sufficient quantity to allow quantitation on-column, its response factors (a, b and c) were taken as the average of all other esters.

Absolute recoveries and recoveries corrected for losses by the recovery standards (CHCA and 3ET) were evaluated in plasma and brain tissue obtained from mice, rats and humans at several spiked levels of analytes. Two sets of recovery experiments were performed for each matrix, except human brain, at nominal analyte concentrations of 0.05 and 2.5 μg/ml, with five to nine replicates. Because of the scarcity of fresh human brain samples, only three replicates at 0.05 μg/ml were done.

The homogeneity and identity of the chromatographic peaks were confirmed by GC–MS using a VG 70-70H double-focusing mass spectrometer (VG Analytical, Manchester, UK), interfaced to a Hewlett-Packard 5710 gas chromatograph and an on-line VG 2035 data system. Splitless injections (3 μl) were made onto a Stabilwax column at a head pressure of 10.3 kPa (helium). The temperature program was 0.5 min at 40°C, then 2°C/min to 250°C. Injector, ion source and interface temperatures were 200, 200 and 250°C, respectively. Ionization energy was 70 eV for electron impact ionization (EI) and 40 eV for chemical ionization (CI). The scan rate was 1 decade/s and the accelerating potential was 4 kV. Methane was the reagent gas.

Correlation of peaks run on GC–ECD with hydrogen carrier gas to those obtained by GC–MS with helium carrier gas was facilitated through use of methylene index units based on an homologous series of 1-chloroalkanes.

EI fragmentation of the PFB esters was nearly identical to the fragmentation of the methyl esters [17], except that the base peak of each PFB ester was m/z 181 $[C_6F_5CH_2]^+$. Similarly, CI gave m/z 181 $[C_6F_5CH_2]^+$ as the base peak but, unlike EI, CI predominantly gave $[M+1]^+$ and $[M]^+$ ions.

3. Results and discussion

3.1. Post-derivatization clean-up

Derivatization of VPA and its metabolites with PFB-Br required clean-up prior to analysis, otherwise excess reagent and side-products created a high background on ECD. Even though much of the PFB-Br was removed by complexation with PPY, enough reagent remained to obscure the chromatogram (Fig. 3A). Several methods of post-derivatization clean-up have been used previously, such as evaporation of excess PFB-Br and side-products [21,24,30], silica gel chromatography on miniature columns [22,28,34] and reaction of excess reagent with a phenolalkylamine [25]. However, the latter method did not remove side-products such as PFB-OH and some PFB-Br remained. Miniature cyanopropyl reversed-phase silica columns have also been used to remove most of the PFB-Br in a derivatization of carboxylic acids [35].

We evaluated evaporation, miniature column chromatography and other clean-up methods for their suitability with our analytes at low concentrations. An attempt was made to reduce chromatographic background by evaporation of the derivatization mixture with a stream of nitrogen. As THF evaporated, a yellow crystalline residue formed. At this point, recoveries were approximately 50%. With prolonged evaporation, the chromatograms improved in appearance but yields decreased to less than 20% after 10 min, due to the volatility of the valproyl PFB esters. To minimize background, it was necessary to evaporate the THF from the reaction mixture prior to the addition of isooctane. THF acted as a

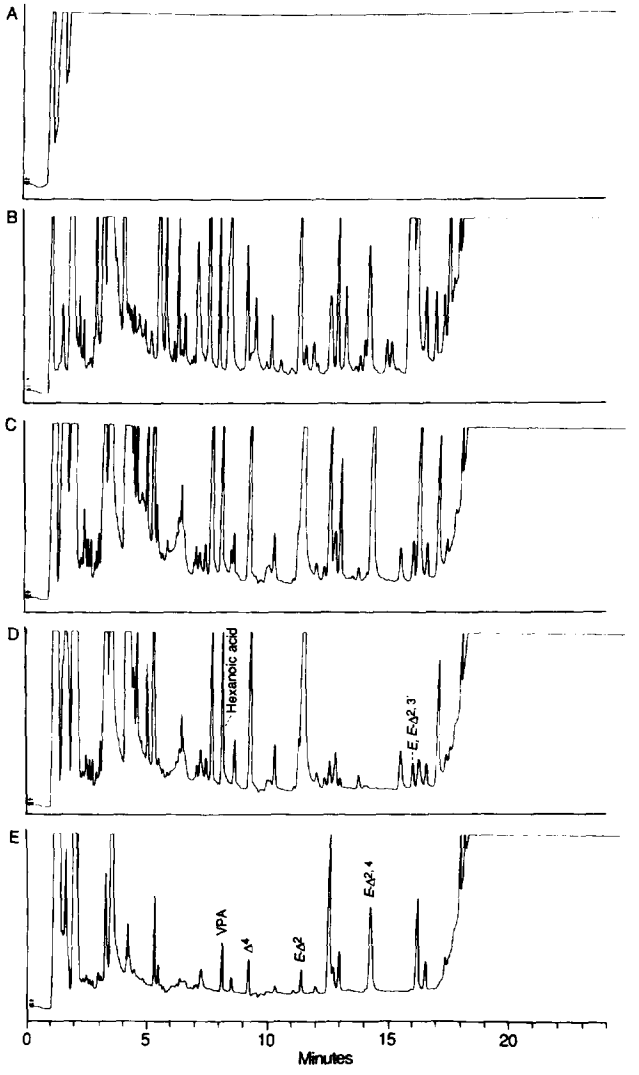

Fig. 3. Gas chromatograms of derivatized extract from spiked mouse plasma using various strategies of clean-up. (A) Post-derivatization mixture after addition of 1 ml of isooctane. (B) The post-derivatization mixture was evaporated to dryness with nitrogen and redissolved in 1 ml of isooctane. This was evaluated as an alternative to HPLC clean-up. (C) Extract after clean-up by HPLC. Excess PFB-Br was removed by HPLC. The chromatographic appearance was greatly improved. However, VPA and Δ^4-VPA could not be quantitated at these levels because of co-eluting peaks. These interferences were eliminated by collecting two fractions on the HPLC (Fig. 2). The first fraction [GC, shown in (E)] contains VPA and all metabolites except E,E-$\Delta^{2,3'}$-VPA. The second fraction [GC, shown in (D)] contains hexanoic acid that co-eluted with VPA and an unknown peak that co-eluted with Δ^4-VPA, if not separated by HPLC.

co-solvent for polar side-products, which were otherwise insoluble in isooctane. Unfortunately, the decrease in background achieved was offset by the loss of analytes during complete evaporation of the THF (Fig. 3B). Evaporative loss of volatile esters was previously noted [22,28].

Partitioning of the derivatization mixture into water at neutral, basic or acidic pH was not successful in reducing background. Miniature silica columns (1 g) have been previously used to clean up derivatizations [22,28,34]. We found that these columns removed the majority of PFB-Br from the derivatization mixture, but enough PFB-Br remained to interfere with chromatography. Also, interfering

side-products were not removed and loss of analytes at the low ng level were significant. Chromatography on miniature LH-20 columns in either adsorption or partitioning modes was attempted without success.

3.2. Optimization of derivatization

Each method of post-derivatization clean-up caused unacceptable losses of analytes. Therefore, we explored conditions that would minimize the derivatization-induced background. Two approaches were tried: (1) minimization of the amount of PFB-Br in the derivatization and (2) reduction of electrophilic side-products through optimization of the derivatization conditions. Others have noted some causes of high background, such as excess PFB-Br [22,24–26,28,34,35], the presence of water [28,29,36] and excess base [27,28,36]. We confirmed, by MS, the presence of the previously identified side-products, polyfluorobenzylethers [27,28,37,36], PFB-OH [36,37] and PFB-Cl [36]. PFB-Br, PFB-OH and PFB-Cl were the primary components of the massive front peaks in the gas chromatogram, which, if not removed, obscured the remainder of the chromatogram (Fig. 3A). The polyfluorobenzylethers, ($C_6F_5CH_2$-[O-$C_6F_5CH_2$-]$_n$-OH), elute as an homologous series of interfering peaks throughout the chromatogram. The intensity of background changed with the reaction solvent and the profile was dependent upon the base. We therefore evaluated a series of solvents and bases for derivatization yield and interference with analytes.

Derivatization yields increased with the polarity of the derivatization solvent. This is consistent with an SN_2 reaction and a polar transition state [38]. THF, acetonitrile and acetone gave yields of approximately 40–80% for VPA, E-Δ^2-VPA and Δ^4-VPA, while toluene and pentane gave yields of 10–30%. Similar polarity effects were reported when methylene chloride was added to acetone during the derivatization of chlorophenols [39]. All grades of acetone were found to contain impurities that interfered with analytes and, thus, were unsuitable for use. Although previously used by others [28,29], acetonitrile reacted with PFB-Br to form many interfering peaks. Unpreserved THF, freshly distilled from sodium benzophenone, produced no interferences. Preservatives in THF were derivatized by PFB-Br and obscured the analytes in the chromatogram. THF was selected as the derivatization solvent because it produced the least interferences and the best yield of PFB esters.

Esterification with benzyl halides requires a base [23]. Both inorganic bases [15] and organic bases [30] have been used. Inorganic bases were stronger, formed more side-products and also must be removed from reaction mixtures prior to GC on capillary columns.

For these reasons, we surveyed a number of organic bases for use in derivatizations. N-Ethylpiperidine was previously used in the synthesis of 5-indole acetic acid PFB ester [30]. Other tertiary amines, such as N,N-diisopropylethylamine, have been used frequently in the derivatization of prostaglandins [40]. Triethylamine was used to derivatize long-chain alkyl carboxylic acids [41]. We found that N,N-diisopropylethylamine, N-ethylpiperidine and triethylamine produced interferences on the GC. Poly(4-vinylpyridine) and 1,2,2,6,6-pentamethylpiperidine were inactive. Unsubstituted pyridine was not active for us or others [30]. The hypernucleophilic catalysts [42], DMAP and PPY, gave the best yields of PFB esters and the fewest interfering peaks. Furthermore, each fortuitously reduced the free level of PFB-Br in the reaction mixture. Addition of DMAP to a solution of PFB-Br in dry THF formed a fine whitish–yellow precipitate, which was the complex, 1-N-pentafluorobenzyl-4-N,N-dimethylaminopyridinium bromide. A reddish oil was formed when PPY was used; the complex in this case was 1-N-pentafluorobenzyl-4-pyrrolidinopyridinium bromide. These complexes were destroyed by moisture. Pyridinium salts are active alkylation species [36] and therefore can participate in alkylation as well as in the reduction of the concentration of free PFB. When HPLC was used to clean up the derivatization mixture, PPY was preferred; DMAP–PFB-Br complex continued to precipitate in the mobile phase, even after lengthy centrifugation of the reaction mixture. PPY–PFB-Br complex was easily removed prior to HPLC by centrifugation after dilution of the derivatization mixture with isooctane.

Water contributed to the derivatization-induced background through several mechanisms. Water reacted with PFB-Br to form PFB-OH and participated in the formation of polypentafluorobenzyl

ethers and other side-products. Because water consumed PFB-Br, an excess of PFB-Br was needed, which further contributed to the background. Water also prevented formation of DMAP–PFB-Br or PPY–PFB-Br complexes, which serve to tie up excess PFB-Br without affecting the derivatization yield.

Two strategies to prevent water from entering the derivatization reaction were examined; drying the solvent extract or using hydrophobic solvents for the extraction. Powdered molecular sieves and magnesium sulfate are among the most effective drying reagents [43]. These reagents caused a 100 and 50% decrease, respectively, in recovery in VPA, E-Δ^2-VPA and Δ^4-VPA. Other drying reagents had incompatibilities with acids, for example, lithium aluminum hydride and calcium hydride, or offered no improvements in efficacy and recovery, for example, sodium sulfate. Lowered recoveries were observed when sodium sulfate was used to dry trace levels of acid herbicides [44].

Because drying the extracts was not viable, we sought to reduce water partitioning into the extraction solvent. Hydrophobic solvents, hexane, pentane, carbon tetrachloride, trichlorotrifluoroethane (Freon 112) and butylchloride were evaluated for extraction efficiency, losses from solvent reduction and interference with derivatization. Comparisons were made between these and more polar solvents, such as chloroform and ethyl acetate. Not unexpectedly, extraction efficiencies for non-polar solvents, such as pentane, were unacceptably low with recoveries less than half of the more polar solvents. There were no significant differences in extraction with solvents of moderate polarity. Extraction recoveries for 4,5-[^{3}H]-VPA were 60–80%. This agreed with previous work with chloroform and VPA [20]. We found that ethyl acetate, a solvent previously used for VPA analysis [12–17], was not significantly better than chloroform or butylchloride for extraction of VPA and its unsaturated metabolites. In our case, ethyl acetate was not a suitable extraction solvent because acetic acid present in even high-quality reagent reacted with PFB-Br and obscured the GC chromatograms.

During evaporation of solvent, additives and polarity of solvent had influenced VPA recovery (Table 2). Nitrogen blow down was an effective method for concentration of most solvent extracts, without loss of valproyl analytes. This agreed with previous work that found that this technique was superior to Kuderna–Danish concentration, rotary evaporation and heated evaporation when using methylene chloride [45]. However, we found that when extremely volatile solvents, such as Freon 112 or pentane, were evaporated with nitrogen, low recoveries resulted. This may have been a physical phenomena; the quickness of evaporation could have left analyte on the side of the tube. Recoveries from these volatile solvents were increased by boiling at reflux in a micro-Kuderna–Danish apparatus (Table 2). Addition of a polar co-solvent to the extraction solvent prior to evaporation, e.g. acetonitrile, did not decrease evaporative losses, in contrast to a study with

Table 2
Recovery of 4,5-[^{3}H]-VPA from extraction solvents during the concentration step

Extraction solvent	Recovery [mean±S.D. (n)]			
	1	2	3	4
Freon 112	0.61±0.15 (10)	0.23±0.08 (5)	0.86±0.15 (10)	0.94±0.12 (7)
Pentane	0.35±0.17 (5)	0.50±0.08 (5)	0.62±0.06 (4)	0.38±0.10 (5)
Chloroform	0.81±0.10 (10)	0.51±0.05 (5)	0.89±0.09 (7)	–
Butylchloride	0.90±0.11 (10)	0.51±0.08 (5)	0.88±0.18 (9)	–
Ethyl acetate	0.90±0.16 (10)	0.40±0.04 (5)	–	–

Experimental conditions: (1) solvent (0.8 ml) was evaporated with a dry N_2 stream to 50 μl in a polypropylene microcentrifuge tube; (2) same as 1 except that 50 μl of acetonitrile were added to the solvent prior to concentration; (3) same as 1 except that 50 μl of DMAP (42 mg/ml in THF) were added to the solvent; (4) solvent (4 ml) was evaporated to 50 μl at reflux in a micro-Kuderna–Danish apparatus.

PFB phenol ethers [39]. Ion pairing of carboxylic acids with DMAP decreased evaporative losses, but proved unsatisfactory because the DMAP–carboxylic acid salts were deliquescent.

The optimum solvent depended on the approach to reduce derivatization background. For Method 1, which minimized background formation through reduction of water in the derivatization, butylchloride (a solvent of moderate polarity, low water adsorption and with a density less than that of water) was selected. The latter property made manipulation of the extract easier. The nature of the solvent was much less critical when HPLC post-derivatization clean-up was used. The solvent merely had to efficiently extract VPA and the metabolites. The amount of PFB-Br was increased to compensate for the water introduced through the solvent extract. Derivatization proceeded to the same extent even with visible amounts of water present.

Derivatization conditions were optimized. Derivatization yield was maximum at 1 h. The optimum temperature for derivatization was approximately 40°C. Above 40°C, yields declined as increasing amounts of side-products were formed. Analytes may have become trapped in polymeric material formed at the higher temperatures or may have reacted with side-products such as polyfluorobenzyl ether bromide. Recovery standards behaved in a similar manner to that of the analytes.

PFB-Br had to be in molar excess over the carboxylic acids present in the extract. The magnitude of the excess depended on whether background was to be reduced through optimization of the derivatization (method 1) or by clean-up (method 2). For method 1, a balance between effects on yield and background was reached at a 200-fold molar excess for 35 nmol of carboxylic acids. For method 2, the amount of PFB-Br was not constrained; a 4800-fold excess allowed for consumption through side reactions. Base concentration had little effect on derivatization yield, provided that at least a stoichiometric amount was present. Therefore, a two-fold excess of base was used.

Method 1 proved to be less than optimal. Chromatographic appearance, assay sensitivity and quantitation were critically dependent upon the derivatization being anhydrous, a troublesome and continual problem. Post-derivatization clean-up was deemed necessary to reduce interferences and to decrease background-limited sensitivity. This led to the development of Method 2.

3.3. HPLC clean-up

Clean-up of the derivatization by normal phase HPLC eliminated constraints on the choice of solvent, the presence of water and the amount of PFB-Br in the derivatization mixture. PFB-Br, PFB-OH and PFB-Cl eluted near the void volume, while the esters began to elute at approximately 4 min (Fig. 2). Alkyl substitution on the α-carbon influenced elution in a predictable manner; among carboxylic acids of similar molecular masses, the elution order was cyclic acids, unsaturated acids and finally saturated acids. By appropriate choice of collection times, VPA was collected free from contamination from hexanoic acid, which co-eluted with VPA on the GC. Hexanoic acid was a ubiquitous acid present in all of our samples at concentrations approaching 100 ng/ml. Recoveries from HPLC for VPA, E-Δ^2-VPA, Δ^4-VPA and E,E-$\Delta^{2,3'}$-VPA PFB esters were greater than 90%. HPLC was used previously to separate PFB esters of long-chain carboxylic acids [46] on silica columns for semi-preparative purification of isomers. Grob et al. [47] used HPLC–GC with a retention gap and pre-detector effluent splitter to remove excess PFB-Br.

PFB-Br reacting with endogenous compounds in the sample also contributed to chromatographic complexity. PFB-Br derivatization of long-chain fatty acids produced large peaks late in the chromatogram and contributed to the late rise in the baseline (Fig. 3C,D), while esterification of small-chain aliphatic acids (C_5–C_{12}) produced a series of peaks in the region where the analytes elute.

3.4. GC analysis

GC columns of varying polarity were evaluated for resolution between the unsaturated metabolites and VPA. Because of the greater than 100-fold difference in concentrations between parent and metabolites, the resolution had to be large. VPA invariably co-chromatographed with one of the

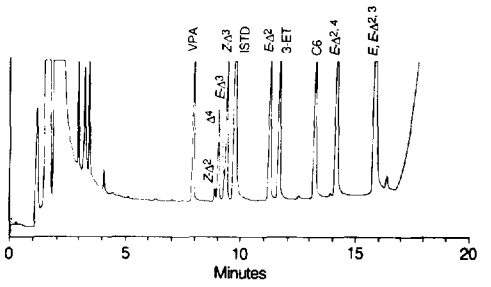

Fig. 4. A 1-μl splitless injection of a 10 ng/ml solution of VPA, its metabolites and recovery standard as PFB esters. Resolution of E-Δ^3-VPA and Z-Δ^3-VPA was dependent on their relative proportions (in this case 1:20) and on the age of the column. All other metabolites were well resolved.

monoene metabolites on low-to-moderate polarity columns, such as DB-1 or DB-1701. We found that the Stabilwax column, which is a polar, oxygen-stabilized carbowax, gave suitable resolution. A very slow temperature ramp (2°C/min) provided an improved separation between the geometrical and structural isomers of the monoene metabolites. Resolution of E-Δ^3-VPA and Z-Δ^3-VPA was dependent on their relative proportions and the age of the column. All other isomers were well resolved (Fig. 4).

3.5. Assay performance

For the purposes of method development and validation, the absolute recoveries of VPA, several of the metabolites and the recovery standards were evaluated. Overall recoveries were 55% for mouse plasma, 38% for mouse brain, 46% for human plasma and 35% for human brain. The recoveries of the recovery standards, 3ET and CHCA, mirrored those of the analytes and, thus, the recovery standards were suitable for these assays.

Calibration curves based on relative response to the recovery standard for the analytes were linear; the S.E. of regression averaged over all compounds were as follows: mouse plasma, 0.256; mouse brain, 0.126; rat plasma, 0.066; rat brain, 0.196; human plasma, 0.188 and human brain, 0.144. The behavior of VPA was not ideal at low levels of analyte, with standard curves having a positive intercept because of variable, low level contamination. Therefore, the lower limit of VPA quantitation in our laboratory was 0.1 μg/ml (or μg/g), based on a 50-μl or 50-mg sample. For the other compounds, we regularly quantitated levels down to 0.02 μg/ml (or μg/g). Further sensitivity was gained through concentration of the HPLC fractions to volumes of less than 1 ml. In this manner, quantitation limits of the order of 0.001 μg/ml (or μg/g) was achieved.

Results of recovery experiments are presented in Table 3. Recoveries based on the recovery standards ranged from 74–124%. The lowest value represents the low level VPA spike that had interference from low level contamination. The high value represents the Δ^4-VPA spike in mouse brain homogenate, which occasionally presented an incompletely resolved interference. Aside from these exceptions, recoveries

Table 3
Performance of the assay after correction for recovery

	Spike concentration (μg/ml, μg/g)	Recovery after correction by recovery standards [mean±S.D. (n)]					
		VPA	Δ^4-VPA	E-Δ^2-VPA	E-$\Delta^{2,4}$-VPA	E,E-$\Delta^{2,3}$-VPA	Average
Mouse plasma	0.05	0.74±0.08 (5)	1.12±0.06 (5)	1.01±0.05 (5)	0.99±0.06 (5)	0.95±0.10 (5)	0.96±0.14
	2.5	0.96±0.08 (5)	1.09±0.09 (5)	1.00±0.08 (5)	1.08±0.07 (5)	1.04±0.22 (5)	1.03±0.05
Mouse brain	0.05	0.98±0.04 (6)	0.95±0.02 (6)	0.93±0.05 (6)	0.93±0.03 (6)	0.93±0.24 (6)	0.94±0.02
	2.5	1.05±0.11 (6)	1.25±0.10 (6)	0.97±0.03 (6)	1.02±0.08 (6)	0.96±0.09 (6)	1.05±0.12
Human plasma	0.05	0.82±0.11 (9)	1.01±0.09 (7)	1.01±0.05 (7)	0.99±0.06 (7)	1.02±0.07 (7)	0.97±0.08
	2.5	0.96±0.09 (7)	1.10±0.10 (7)	1.09±0.12 (7)	1.13±0.12 (7)	0.84±0.08 (7)	1.02±0.12
Human brain	0.05	0.93±0.15 (3)	0.95±0.05 (3)	1.04±0.05 (3)	0.88±0.07 (3)	0.99±0.05 (3)	0.96±0.06
Rat plasma	0.25	1.04±0.11 (10)	0.94±0.07 (10)	0.91±0.10 (10)	1.12±0.10 (10)	0.93±0.09 (10)	0.99±0.09
Rat brain	0.05	1.08±0.11 (10)	1.06±0.10 (10)	0.91±0.11 (10)	1.02±0.10 (10)	0.97±0.10 (10)	1.01±0.07

for the compounds in four matrices averaged about 100±15%. The coefficients of variation for the assay of these compounds were usually less than 11%.

3.6. Assay interferences

The three methods presented had interferences, some of which were eliminated by the use of HPLC. Hexanoic acid was not resolved from VPA on the GC with any of the phases tested. By use of the HPLC method for clean-up, we were able to eliminate this source of interference (Fig. 3D,E). We were unable to eliminate the low level (approximately 0.02 μg/g) contamination by external sources of VPA, as the volatile compound was used in high concentrations in the laboratory. On occasion, an unidentified compound in mouse brain samples was not completely resolved from Δ^4-VPA. This caused an overestimation of Δ^4-VPA concentration by approximately 20% at low concentrations (approximately 0.05 μg/ml). GC–MS demonstrated that other analyte peaks were homogeneous, as analytes in rat brain, mouse brain, human brain, and rat plasma had no interferences. Human plasma samples from patients on a variety of anticonvulsant drugs, including carbamazepine, phenytoin, clorazepate and nordiazepam, were evaluated and showed no interferences. The convulsant, pentylenetetrazole, did not cause interferences after administration to animals. During our examination of peak homogeneity, we noticed that, in the human brain samples, two resolved peaks near VPA and E-Δ^2-VPA were PFB esters of branched-chain acids of similar molecular mass to VPA. Unfortunately, positive identification could not be made with either EI or CI GC–MS.

In conclusion, while careful control of reaction conditions can minimize the amount of background in derivatizations with PFB-Br, this approach was not as reliable as post-derivatization clean-up by HPLC.

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