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RESOLUTION OF VALPROIC ACID FROM DEUTERATED ANALOGUES AND THEIR QUANTITATION IN PLASMA USING CAPILLARY GAS CHROMATOGRAPHY

D.J. HOFFMAN*

Pharmaceutical Products Division, Abbott Laboratories, North Chicago, IL 60064 (U.S.A.)

and

W.R. PORTER

School of Pharmacy, University of Wisconsin, Madison, WI 53706 (U.S.A.)

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SUMMARY

Quantitation of valproic acid and a deuterated analogue in the same plasma sample by capillary gas chromatography without mass spectrometry was illustrated. Specificity was accomplished solely with a 60 m × 0.25 mm fused silica WCOT column coated with OV-351. A hexadeutero and two tetradeutero analogues of valproic acid had resolutions of at least 1.2 from valproic acid. Plasma samples were extracted with carbon tetrachloride following the addition of 2-ethylhexanoic acid as the internal standard. The method is sensitive to at least 0.5 μg/ml and provides the capability of conducting absolute bioavailability and pulsed dosing studies with deuterated drug analogues without a mass spectrometer. The technique was applied to the analysis of plasma samples from dogs simultaneously administered valproic acid and a deuterated analogue.

INTRODUCTION

The merits of stable isotopes in biomedical research has recently been reviewed [1—3]. Major applications in the pharmaceutical area include absolute bioavailability, bioequivalence and pharmacokinetics in chronic drug administration (pulse dosing). The elimination kinetics of a tetradeutero valproic acid isomer was recently studied in chronic epileptic patients on a maintenance dose of valproic acid [4]. Deuterium is the most extensively used heavy stable isotope primarily due to its ready availability, low cost, and high isotopic purity (> 99%). Because of the primary and secondary isotope

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effects associated with many deuterium analogues [3], the use of more costly ^{13}C or ^{15}N analogues has been recommended [1]. However, deuterium may be used provided it is located in a metabolically inert region of the molecule to ensure the absence of significant isotope effects.

The only method currently available to quantitate both the light and heavy isotopes in the same sample is gas chromatography—mass spectrometry (GC—MS). Both conventional and capillary GC systems are used. It has been noted in our laboratory and by Durden and Boulton [5] using a capillary GC—MS system that deuterated and unlabeled analogues are not isographic; deuterated analogues elute a few seconds prior to the unlabeled compounds. In this paper we demonstrate the capability of capillary GC to resolve and quantitate unlabeled from deuterated compounds without MS. The technique is applied to the simultaneous analysis of the anticonvulsant valproic acid and deuterated analogues in dog serum.

EXPERIMENTAL

Chemicals and reagents

Valproic acid (VPA) was obtained from Abbott Laboratories. 4,4',5,5'-d_4-Valproic acid (I) was synthesized by reducing diallylacetic acid with deuterated hydrazine hydrate (MSD Isotopes, Dorval, Canada) [6]. The 3,3,3',3'-tetradeutero- (II) and 5,5,5,5',5',5'-hexadeuterovalproic acid (III) were purchased from MSD Isotopes. All deuterated valproic acids were free of valproic acid (< 0.15%) as determined by capillary GC. Assigned structures were in agreement with their respective NMR, IR and mass spectra.

Stock aqueous standard solutions of about 1 mg/ml were prepared by dissolving about 50 mg of the acid in 1 ml of 1 *N* sodium hydroxide and adjusting to 50 ml with distilled water. Aqueous standards were stored below 10°C. Plasma standards containing both a deuterated valproic acid and valproic acid in ratios of 0.5 to 2.0 were prepared by appropriate dilution of the stock solutions with fresh bovine plasma to the concentration range of 0.1—50 μg/ml. Internal standard, 2-ethylhexanoic acid (Aldrich, Milwaukee, WI, U.S.A.), was prepared at 150 μg/ml in water.

Carbon tetrachloride was HPLC grade. All other reagents were analytical reagent grade.

Gas chromatography

A fused silica capillary column (60 m × 0.25 mm I.D.) coated with OV-351, film thickness 0.25 μm (J & S Scientific, Crystal Lake, IL, U.S.A.), was used with a Hewlett-Packard Model 5840A gas chromatograph equipped with an autosampler. The injection port containing a 8 cm × 2 mm I.D. fused silica splitless insert was kept at 220°C, the column was operated isothermally at 155°C and the flame ionization detector temperature was 240°C. Hydrogen carrier gas was used at a flow-rate of 41 cm/sec with a split ratio of about 10:1. All three deuterated valproic acids were resolved (resolution > 1.2) from VPA and internal standard, 2-ethylhexanoic acid (Fig. 1).

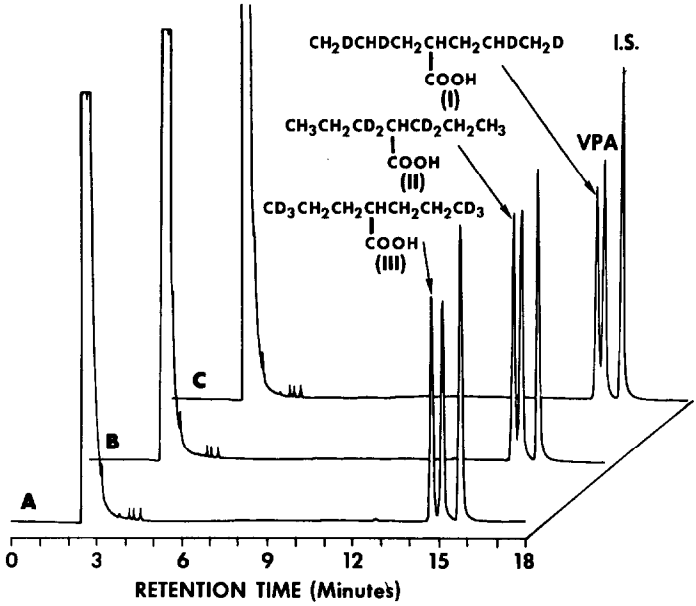

Fig. 1. Chromatograms of plasma extracts of spiked standards. Plasma standards containing: (A) VPA (25.9 μg/ml), III (27.1 μg/ml) and internal standard; (B) VPA (27.1 μg/ml), II (28.0 μg/ml), and internal standard; (C) VPA (23.5 μg/ml), I (24.6 μg/ml), and internal standard.

Assay procedure

A 1-ml plasma sample or standard and 0.2 ml of internal standard solution were added to a 12-ml conical screw cap test tube. A 0.2-ml volume of 22.5% perchloric acid was added and mixed by vortexing. Carbon tetrachloride (0.4 ml) was added followed by vortexing for 10 sec and centrifugation at 1800 *g* for 5 min. The upper aqueous phase was discarded by aspiration leaving a white protein plug above the carbon tetrachloride layer. A Pasteur pipet was used to penetrate the protein layer and cleanly withdraw and transfer the clear carbon tetrachloride to a 0.3-ml microvial. After capping the microvial a 2-μl aliquot was automatically injected into the gas chromatograph. All samples and standards were randomly mixed prior to injection.

Plasma standards of known concentrations of labeled and unlabeled valproic acids were used to construct calibration curves. Three calibration curves were initially constructed for each acid; one curve each for labeled/unlabeled ratios of 2:1, 1:1 and 1:2. Because the calibration curve parameters (slope and intercept) of all three calibration curves were practically identical, in each case the data from the three ratios were combined into a single calibration curve which was used to estimate plasma unknowns. Calibration curve parameters from a plot of peak area ratio (acid/internal standard) versus standard concentrations were obtained by least-squares linear regression analysis. Other than plasma blanks, plasma standard concentrations ranged from about 0.1 μg/ml to 75 μg/ml.

Drug administration to dogs

Fasted beagle dogs weighing 8—10 kg were orally administered 10 ml of a buffered bicarbonate—carbonate (0.025 *M*, pH 9—9.5) solution containing approximately 100 mg each of valproic acid and a deuterated analogue. Actual amounts were adjusted so that equal molar equivalents were administered. Heparinized 5-ml blood samples were obtained prior to dosing and at specified times post-administration. The plasma was isolated into labeled test tubes and held frozen until assayed.

RESULTS AND DISCUSSION

In order to achieve a resolution of at least 1.25 for two components with an α-value of 1.02 a chromatographic column requires a total of at least 65,000 effective theoretical plates [7]. This degree of efficiency, which is necessary to separate an unlabeled component from the same component labeled with deuterium, can be achieved using narrow-bore WCOT capillary GC columns. In this study, two tetradeutero and one hexadeutero analogue of VPA were resolved from unlabeled VPA on a 60 m × 0.25 mm I.D. WCOT fused silica column coated with OV-351 (film thickness 0.25 μm).

The resolutions of I, II and III from VPA at various oven temperatures are shown in Table I. The choice of 155°C was a compromise between resolution and analysis time. The degree of resolution is primarily dependent upon the deuterium content of the molecule. However, the position of the deuterium atoms has a small effect on the chromatographic properties. This is illustrated with the two tetradeutero VPA analogues (I and II) which are positional isomers and have slightly different retention times (Table I).

A small amount of tailing occurs when the underivatized acids are chromatographed (Fig. 1). Tailing factors were kept below 1.3 by occasional cleaning of the fused silica splitless liner. Various split liner insert combinations were examined which contained silylated glass wool or GC packing material coated with 5% FFAP. All split inserts had a detrimental effect on chromatographic efficiency due to increased peak tailing. Using a clean splitless insert, significant tailing never became a problem. If tailing had become

TABLE I

RESOLUTION OF DEUTERATED VPA ANALOGUES FROM VPA AS A FUNCTION OF TEMPERATURE

Hydrogen flow-rate 41.5 cm/sec; resolutions (R_s) are the mean of three determinations; t_R = retention time.

Oven temperature (°C)	I		II		III		VPA
	t_R (min)	R_s	t_R (min)	R_s	t_R (min)	R_s	t_R (min)
145	21.80	1.41	21.75	1.64	21.61	2.25	22.18
150	17.96	1.35	17.92	1.55	17.84	2.17	18.33
155	14.82	1.21	14.79	1.39	14.70	1.96	15.19
160	13.39	1.09	13.36	1.16	13.20	1.74	13.52
Deuterium content (%)	5.40		5.40		7.99		—

TABLE II

CALIBRATION CURVE SLOPES FOR VARIOUS RATIOS OF LABELED/UNLABELED VPA ANALOGUES

Analogue	Ratio (Labelled/unlabelled)	Calibration curve slopes*	
		Deuterated VPA	VPA
I	2:1	0.0312	0.0376
	1:1	0.0310	0.0365
	1:2	0.0305	0.0349
Combined (n=21)		0.0310	0.0358
II	2:1	0.0340	0.0353
	1:1	0.0316	0.0352
	1:2	0.0328	0.0380
Combined (n=21)		0.0331	0.0357
III	2:1	0.0322	0.0361
	1:1	0.0315	0.0345
	1:2	0.0330	0.0360
Combined (n=21)		0.0321	0.0356
None			0.0353

*Individual curves have n=7 points; correlation coefficients were > 0.99 for all curves.

TABLE III

QUANTITATION OF VPA AND *d*-VPA ANALOGUES AT HIGH UNLABELED/LABELED RATIOS

Samples estimated from a calibration curve which had standards at a 1:1 ratio.

Deuterated analogue	Concentration (μg/ml)	Ratio* (VPA/*d*-VPA)	Calculated concentration (μg/ml)	
			d-VPA	VPA***
I	0.97	54:1	0.91 ± 0.10 (11.0)**	53.8 ± 1.8 (3.5)
	30.5	1.7:1	31.4 ± 1.6 (5.1)	51.9 ± 1.9 (3.7)
II	1.02	52:1	0.95 ± 0.09 (9.8)	52.4 ± 2.1 (4.0)
	31.4	1.7:1	31.8 ± 1.4 (4.5)	53.2 ± 2.0 (3.8)
III	0.95	55:1	0.92 ± 0.09 (10.1)	53.1 ± 1.3 (2.4)
	30.4	1.7:1	29.8 ± 1.8 (6.1)	52.5 ± 1.8 (3.4)

*Each ratio determined in quadruplicate.
**Coefficient of variation in parentheses.
***Actual VPA concentration was 52.7 μg/ml.

a problem, esterification would have been required to produce symmetrical peaks. A split ratio of 10:1 provided high sensitivity without significant loss of efficiency.

The extraction process, which was a modification of the method of Dijkhuis and Vervloet [8], was rapid and provided chromatograms free of interfering

peaks (Fig. 1). Calibration curves for the three deuterated acids and VPA were linear to at least 50 μg/ml. The slope parameters for plasma standard combinations of labeled VPA/unlabeled VPA in ratios of 2:1, 1:1 and 1:2 did not significantly differ as shown in Table II. For this reason a single calibration curve from a combination of the three ratio curves was constructed to determine unknown samples. The higher slopes of the VPA calibration curves are primarily due to a slightly lower response of the detector for the deuterated analogues. Because I and II have slightly tailing peaks and are not completely resolved from VPA, a small peak area contribution to the VPA area may also occur. However, analysis of variance indicates no significant difference between the VPA slopes of each analogue suggesting little or no area contribu-

TABLE IV

PRECISION FOR THE DETERMINATION OF VPA AND DEUTERATED ANALOGUES SPIKED IN SERUM

Compound	Serum concentration (μg/ml)			
	Actual	Calculated ± S.D.	*n*	C.V. (%)
4,4',5,5'-d_4-VPA (I)	0	0		—
	0.36	0.35 ± 0.069	3	19.8
	1.79	1.80 ± 0.133	6	7.4
	3.58	3.64 ± 0.350	3	9.6
	8.94	9.25 ± 0.471	3	5.1
	17.89	17.37 ± 1.224	6	7.0
	26.84	26.65 ± 0.692	3	2.6
	35.78	36.36 ± 2.572	5	7.1
3,3,3',3'-d_4-VPA (II)	0	0		—
	0.26	0.25 ± 0.045	4	18.1
	1.75	1.76 ± 0.101	7	5.7
	2.62	2.63 ± 0.102	4	3.9
	8.74	8.89 ± 0.429	3	4.8
	17.49	17.75 ± 0.684	7	3.8
	26.23	26.13 ± 1.309	4	5.0
	34.97	35.56 ± 1.740	5	4.9
5,5,5,5',5',5'-d_6-VPA (III)	0	0		—
	0.27	0.25 ± 0.052	3	20.9
	1.81	1.75 ± 0.176	5	10.0
	3.62	3.35 ± 0.183	3	5.1
	9.05	8.90 ± 0.400	3	4.5
	18.10	18.07 ± 0.561	6	3.1
	27.15	25.72 ± 0.960	3	3.7
	36.20	35.88 ± 0.492	5	1.4
VPA	0	0		—
	0.34	0.34 ± 0.039	3	11.5
	1.71	1.74 ± 0.099	8	5.7
	3.42	3.36 ± 0.145	4	4.3
	8.54	8.93 ± 0.631	3	7.1
	17.09	17.61 ± 0.850	8	4.8
	25.63	25.48 ± 0.375	5	1.5
	34.17	34.11 ± 0.705	5	2.1

TABLE V

COMPARISON OF DOG PLASMA SAMPLES ASSAYED BY CAPILLARY AND CONVENTIONAL GC

Dog was orally administered 200 mg of valproic acid.

Sample time (min)	VPA concentration* (μg/ml)	
	Capillary GC	Conventional GC
0	0	0
15	69.8	74.6
30	39.1	49.2
45	32.6	34.3
60	25.4	22.5
80	17.1	17.5
100	13.6	13.2
120	11.3	10.9
150	8.8	7.9
180	7.2	7.1
210	6.7	6.4
240	7.8	7.2
300	6.8	6.6
360	4.0	3.6

*Each value is the average of two determinations.

tion by preceding deuterated peaks. All intercepts were not significantly different from zero.

Unlabeled/labeled ratios of 50 and greater are encountered in pulse dose studies in which patients on a maintenance dose of unlabeled drug are administered a single dose of labeled drug [4]. The accuracy and precision of this method is good when applied to plasma samples containing high unlabeled/labeled ratios as shown in Table III. The calibration curves used to estimate the high ratio samples were constructed from standards having unlabeled/labeled ratios of 1:1.

Assay precision is given in Table IV. The deuterated analogues were assayed in the presence of various ratios of VPA. Coefficients of variation were consistently below 10% for concentrations above 1.7 μg/ml. The overall average coefficient of variation (C.V.) for all compounds in the 1.7—37 μg/ml range is 5%. Using the described assay conditions the minimum detectable concentration for all compounds is 0.08 μg/ml. The minimal quantitative concentration is about 0.5 μg/ml. The coefficient of variation at this concentration averages about 15% for the three analogues.

The capillary GC method was compared to a conventional GC method [9] by using both methods to assay plasma samples from a dog orally administered 200 mg of valproic acid. Two assays were conducted with each method on separate days and the average results (Table V) are in good agreement.

Dog samples

The samples from each dog were randomized and assayed under blinded conditions. The plasma concentrations of unlabeled and labeled VPA were similar for all three deuterated analogues suggesting no pronounced isotope

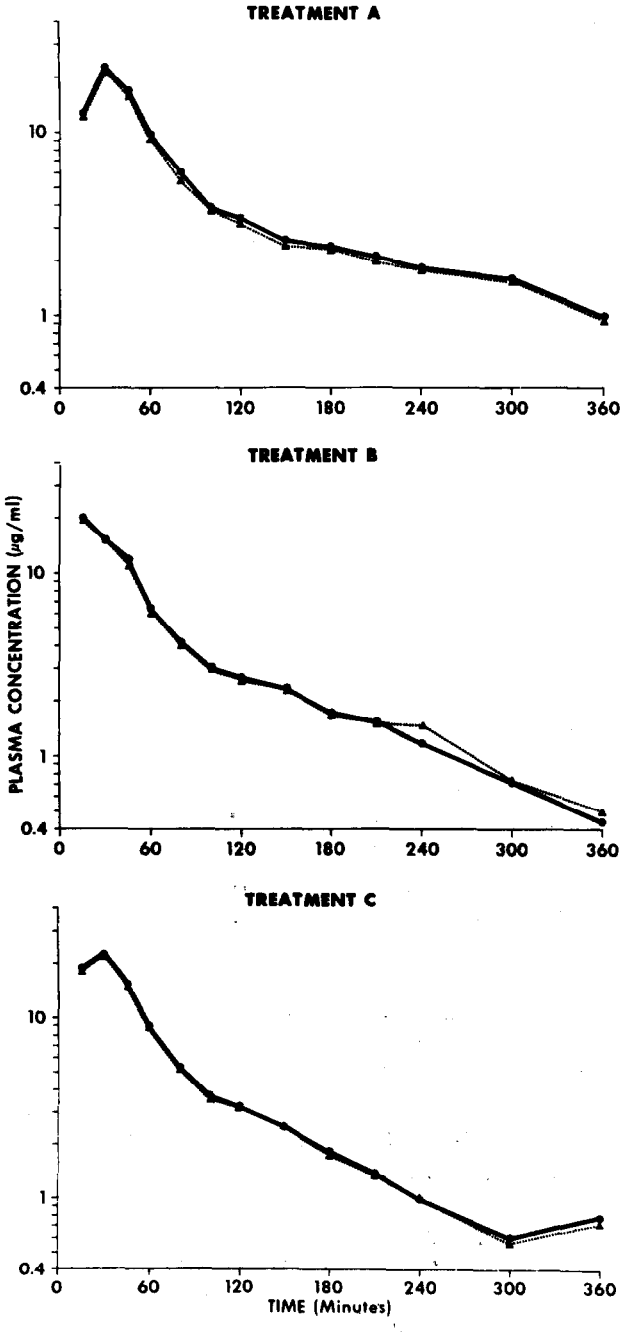

Fig. 2. Plasma concentration vs. time profiles of three dogs simultaneously administered VPA and a deuterated VPA analogue. Treatment A, 104.0 mg of II (●——●) plus 99.7 mg VPA (▲ · · · ▲) to Dog No. 2; Treatment B, 102.8 mg of I (●——●) plus 99.6 mg VPA (▲ · · · ▲) to Dog No. 3; Treatment C, 104.3 mg of III (●——●) plus 102.0 mg VPA (▲ · · · ▲) to Dog No. 6.

effect. The plasma concentration–time curves (Fig. 2) of the deuterated analogues are almost identical to those of VPA even to the last sample time (6 h). This was somewhat surprising since VPA is extensively metabolized [10].

The major advantage of this technique is the elimination of a costly mass spectrometer for certain stable isotope applications. Also, the use of conventional GC detectors (e.g., flame ionization detector, alkali flame ionization detector and electron-capture detector) are less susceptible than a mass spectrometer to sample contamination and maintenance problems. The disadvantages of this technique are that it is probably limited to deuterated analogues and that the chromatography is 2–3 times longer than GC–MS. However, deuterated analogues are satisfactory for bioavailability or pulse dosing studies provided they are labeled, if possible, in metabolically inert positions. The increased analysis time is not a major problem with modern instrumentation, which have automatic samplers and stable detection systems. Because of the good stability of this system, up to 60 VPA samples were assayed daily on a routine basis.

Resolution (R_s = 1.4) has currently been achieved in our laboratory with an experimental drug having a deuterium content of only 3%. Achieving resolution at even lower deuterium contents will further increase the flexibility of the technique.

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