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Assay for valproic acid and its E-Δ^2 metabolite in rat plasma by capillary gas chromatography without prior derivatization

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Abstract

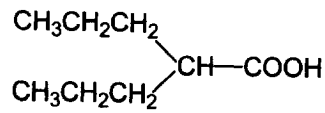
A new and improved gas chromatographic assay method for valproic acid and a metabolite, E-Δ^2 valproic acid, in rat plasma has been developed. The assay has sufficient sensitivity to measure free levels of the parent drug and metabolite. By employing a Stabilwax-DA capillary column, symmetrical chromatographic peaks were obtained without the need for prior derivatization. Standard curves for valproic acid were linear from 0.1 to 640 μg/ml. Standard curves for the metabolite were linear from 0.1 to 556 μg/ml.

Keywords: Valproic acid

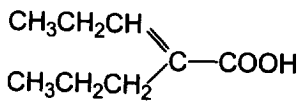
1. Introduction

Valproic acid (2-propylpentanoic acid, VPA) (Fig. 1), has been used to treat seizure disorders since approval by the US Food and Drug Administration in 1978 [1]. The drug is extensively metabolized and the major unsaturated metabolite, E-Δ^2 VPA, is active [2].

Since this metabolite is also less toxic than the parent compound [3–5], it might eventually replace valproic acid. Therefore, we wished to find a rapid method that would quantitate both compounds, and, since the method was to be used in a phar-



VALPROIC ACID



E-Δ² METABOLITE OF VALPROIC ACID

Fig. 1. Structures of valproic acid and the E-Δ^2 metabolite of valproic acid.

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macokinetics study, would have sufficient sensitivity to determine free as well as total drug levels. Since VPA and the E-Δ^2 metabolite are 90% and 99% bound to plasma proteins, respectively [6], the method would have to be capable of quantitating blood levels as low as 0.5 μg/ml. Chromatography of the analytes without prior derivatization would significantly simplify the method and thus shorten the analysis time. There are existing HPLC methods that offer sufficient sensitivity but require prior derivatization of the drug substances to add either a chromophore or a fluorophore [7–10]. A published method for valproic acid without prior derivatization lacked the requisite sensitivity of 0.5 μg/ml as did GC methods using packed columns [11–25].

The high efficiency of capillary GC, however, did afford 0.5 μg/ml sensitivity. A published method derivatizes the carboxyl group to prevent peak tailing [2]. However, we found we could eliminate the derivatization step, thus simplifying the method, by using a Stabilwax-DA column which gave sharp, symmetrical peaks for free carboxylic acids. Because of the satisfactory performance of this column, we did not evaluate other columns.

2. Experimental

2.1. Reagents and supplies

Valproic acid was obtained from Sigma (St. Louis, MO, USA), E-Δ^2 valproic acid from Abbott Laboratories (North Chicago, IL, USA). The identities were confirmed by GC-MS. Cyclohexanecarboxylic acid (internal standard) was obtained from Aldrich (Milwaukee, WI, USA), chloroform (glass distilled, preserved with 1% ethanol) from EM Science (Gibbstown, NJ, USA), and methanol (HPLC grade) from J.T. Baker (Phillipsburg, NJ, USA). All reagents were used without further purification. Rat plasma was obtained from Hilltop Lab Animals (Scottdale, PA, USA).

2.2. Equipment

The gas chromatograph was a Hewlett-Packard Model 5890 equipped with a flame-ionization detector and 3396A integrator. The column was a Stabilwax-DA capillary, 15 m×0.25 mm I.D. with a 0.25 μm film (Restek, Bellefonte, PA, USA). A 5 m retention gap of deactivated fused-silica was attached to the head of the column. Extractions were carried out in 13×100 mm glass tubes fitted with Polyseal (polyethylene lined) caps using a Thermolyne Speci-Mix Model M-26125 and the extracts concentrated in 0.3 ml tapered vials (Alltech Assoc., Deerfield, IL, USA) while heated at 40°C in a Pierce Reacti-Therm III Heating Module (Pierce, Rockford, IL, USA).

2.3. Chromatographic conditions

Hydrogen was used as carrier gas at 0.104 MPa head pressure. The make-up gas was also hydrogen. Splitless injection was employed with a splitless sampling time of 0.75 min. The injector and detector temperatures were 325°C and 275°C, respectively.

The oven temperature program was: initial temp. 45°C, hold 0.75 min, then ramp at 40°C/min to 150°C and hold until the internal standard is eluted. Further ramp at 70°C/min to 240°C and hold until no more peaks are eluted (ca. 12 min total run time).

2.4. Preparation of spiked rat plasma samples

Rat plasma was spiked with valproic acid and the E-Δ^2 metabolite using methanolic solutions. Three concentration series were prepared. Valproic acid concentrations were: Series (1): 0.1, 0.3, 0.5, 0.7, 0.9 and 1.0 μg/ml; Series (2) 10× the concentrations of Series 1; Series (3) 9.9, 34, 51, 85, 113, 142, 163 and 640 μg/ml. The E-Δ^2 metabolite concentrations were ca. 40% of the valproic acid concentrations except for the highest point (556 μg/ml).

2.5. Assay procedure

A 75-μl volume of spiked rat plasma was pipetted into a 13×100 mm glass tube. A 0.4-ml volume of water followed by 20 μl of a methanolic solution of cyclohexanecarboxylic acid as internal standard were added. The concentration of the internal standard solution was 0.024 mg/ml for the lowest concentration range (Series 1), 0.24 mg/ml for the mid range (Series 2), and 2.4 mg/ml for the high range (Series 3). A 100-μl volume of 0.5 M sulfuric acid and 0.5 ml of water-saturated chloroform were

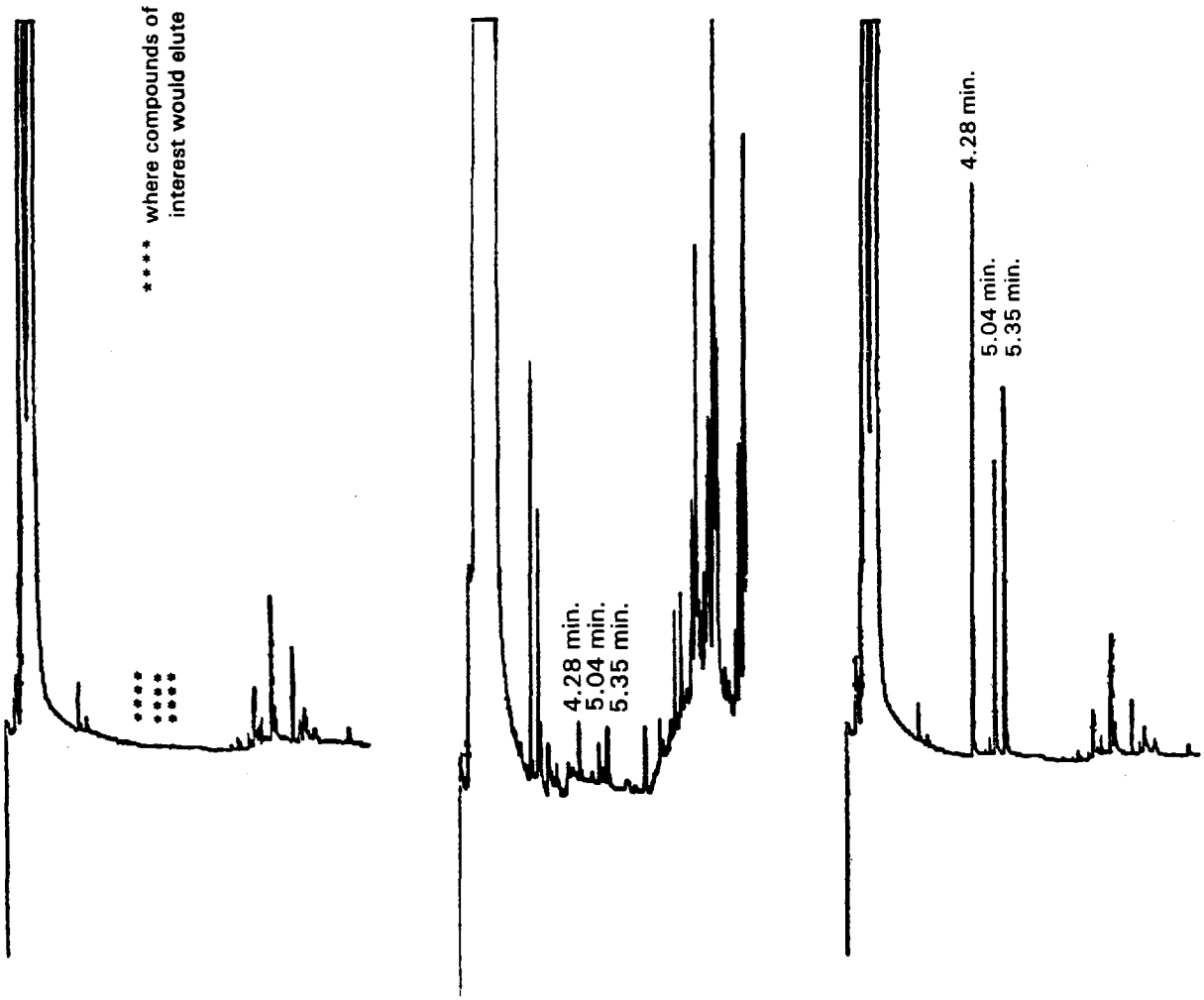

Fig. 2. Chromatograms of rat plasma blank (left), rat plasma spiked with valproic acid and the E-Δ^2 metabolite at concentrations of 0.1 and 0.04 μg/ml, respectively (center), and rat plasma spiked with valproic acid and the E-Δ^2 metabolite at concentrations of 163 and 64 μg/ml, respectively (right). Peak identification according to retention time: 4.28 min, valproic acid; 5.04 min, the E-Δ^2 metabolite; 5.35 min, cyclohexanecarboxylic acid (internal standard).

added, the tube capped with a Polyseal cap and the mixture rocked on a Thermolyne Speci-Mix for 15 min. The mixture was then centrifuged at 2000 *g* for 5 min and the top aqueous phase removed and discarded. The chloroform extract was transferred to a small tapered vial (ca. 0.3–0.4 ml was recovered) and the extract concentrated to ca. 5–50 μl with the aid of a gentle stream of dry nitrogen while maintaining the vial at 40°C in a Pierce Reacti-Therm heating module[1]. A 1–2 μl volume was then injected into the gas chromatograph.

3. Results

3.1. Chromatography

Typical chromatograms are presented in Fig. 2 for a plasma blank (left) and plasma spiked with low

[1] The extract must not be allowed to go to dryness or the analytes will be lost.

Table 1
Valproic acid in rat plasma; intra-day assay precision

Spiked concentration (μg/ml)	Observed concentration (μg/ml)	Mean (μg/ml)	S.D. (μg/ml)	C.V. (%)	Mean error (%)
0.30	0.22	0.26	0.076	29.3	13.8
	0.25				
	0.37				
	0.20				
1.0	1.02	0.94	0.0059	6.3	−8.4
	0.92				
	0.88				
	0.93				
3.0	2.8	2.8	0.22	7.7	−6.7
	2.9				
	2.5				
	3.0				
30.4	32.3	33.6	0.72	2.2	11.3
	33.6				
	33.8				
	33.8				
81.0	76.4	78.8	4.60	5.8	−1.4
	78.7				
	74.7				
	85.2				
126	134	128	5.0	3.9	1.6
	122				
	129				
	127				

Table 2
E-Δ^2 metabolite in rat plasma; intra-day assay precision

Spiked concentration (μg/ml)	Observed concentration (μg/ml)	Mean (μg/ml)	S.D. (μg/ml)	C.V. (%)	Mean error (%)
0.26	0.23	0.29	0.083	29.0	10.2
	0.27				
	0.41				
	0.24				
0.89	0.95	0.87	0.052	5.9	1.7
	0.87				
	0.84				
	0.84				
2.7	2.5	2.9	0.34	12.0	7.0
	3.3				
	2.7				
	2.9				
26.6	25.2	26.5	1.03	3.9	−0.4
	26.4				
	26.7				
	27.7				
71.0	65.3	65.7	3.59	5.5	−7.5
	65.2				
	61.8				
	70.5				
110	111	109	2.36	2.2	0.14
	106				
	111				
	109				

Table 3
Valproic acid in rat plasma; inter-day assay precision (peak area ratios)

Concentration (μg/ml)	Day 1	Day 2	Day 3	Day 4	Average (C.V., %)
0.1	0.275	0.264	0.164	0.356	0.264 (29.7)
0.3	0.425	0.581	0.396	0.411	0.453 (19.0)
0.5	0.839	0.793	0.783	0.760	0.794 (4.2)
0.8	0.880	–	1.00	0.942	0.941 (6.4)
1.0	1.11	1.12	1.24	1.21	1.17 (5.5)

Concentration (μg/ml)	Day 1	Day 2	Average
1.0	0.083	0.145	0.114
4.0	0.583	0.642	0.613
8.0	1.13	1.16	1.15
10.2	1.17	1.34	1.26
12.5	1.48	1.53	1.51

Concentration (μg/ml)	Day 1	Day 2	Day 3	Day 4	Average (C.V., %)
14.7	0.182	0.189	0.207	0.185	0.191 (5.9)
30.4	0.451	0.462	0.414	0.394	0.430 (7.4)
126	1.46	1.48	1.49	1.61	1.51 (4.4)
320	4.40	4.52	4.29	4.26	4.37 (2.7)
640	7.65	7.50	8.93	8.77	8.21 (9.0)

Table 4
E-Δ^2 metabolite in rat plasma; inter-day assay precision (peak area ratios)

Concentration (μg/ml)	Day 1	Day 2	Day 3	Day 4	Average (C.V., %)
0.1	0.210	0.231	0.142	0.277	0.215 (26.1)
0.3	0.371	0.530	0.390	0.382	0.418 (17.9)
0.5	–	0.613	0.635	0.457	0.568 (17.1)
0.8	0.678	–	0.858	0.637	0.724 (16.2)
1.0	0.951	0.901	1.04	0.941	0.958 (6.1)

Concentration (μg/ml)	Day 1	Day 2	Average
0.90	0.071	–	0.071
4.2	0.450	0.533	0.492
7.0	0.812	0.906	0.859
8.9	0.974	1.13	1.05
10.8	1.04	1.17	1.11

Concentration (μg/ml)	Day 1	Day 2	Day 3	Day 4	Average (C.V., %)
12.8	0.152	0.149	0.153	0.146	0.150 (2.1)
26.6	0.353	0.361	0.325	0.293	0.333 (9.3)
110	1.13	1.12	1.12	1.20	1.14 (3.4)
278	3.38	3.49	3.30	3.23	3.35 (3.3)
556	6.27	6.19	6.79	6.72	6.50 (4.7)

(center) and high (right) levels of valproic acid and metabolite. The peaks are base-line separated with no interferences from endogenous substances.

3.2. Intra-day assay precision

New spiked samples were prepared at six levels of valproic acid and the metabolite [two levels in the Series (1) range, one level in the Series (2) range and three levels in the Series (3) range] and assayed by the method above. The results are presented in Tables 1 and 2.

3.3. Inter-day assay precision

Standard extraction curves for the lowest and highest concentration ranges were repeated on four separate days for valproic acid and the metabolite. The curves were repeated on two separate days in the middle concentration range. The data are presented in Tables 3 and 4. Each days results were averaged and regression lines plotted for each concentration range. These are presented in Fig. 3. The lower sensitivity of the FID for the metabolite is probably due to the unsaturated carbon (one less C–H bond than the parent).

3.4. Extraction efficiency

The extraction efficiencies for valproic acid and the internal standard were 95–100%.

3.5. Stability on storage–freeze thaw

Rat plasma spiked with valproic acid at the 56 μg/ml level was stored at −20°C, protected from light, and assayed periodically over a 2-week period. No significant change in valproic acid concentration was noted indicating that neither the storage period nor the freeze thaw cycles caused any loss.

4. Conclusion

The elimination of a derivatization step in our method resulted in a far simpler assay than presented in other published methods. Its simplicity makes it particularly useful in clinical studies where many samples must be assayed and its precision and accuracy are sufficient to make it useful in protein binding studies where very low concentrations of free drug must be measured. The high efficiency of capillary gas chromatography allows compounds of similar structure to be easily separated. In the present application, valproic acid is easily separated from a metabolite which differs from the parent by only a double bond.

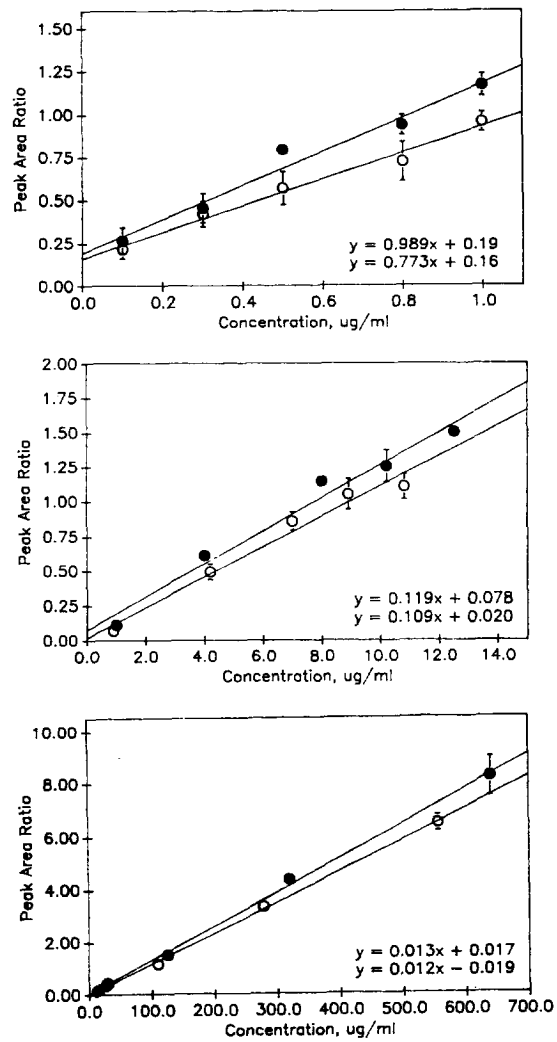

Fig. 3. Regression lines for valproic acid (●) and E-Δ^2 valproic acid (○) extracted from rat plasma. The points are the averages of three runs on separate days.

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