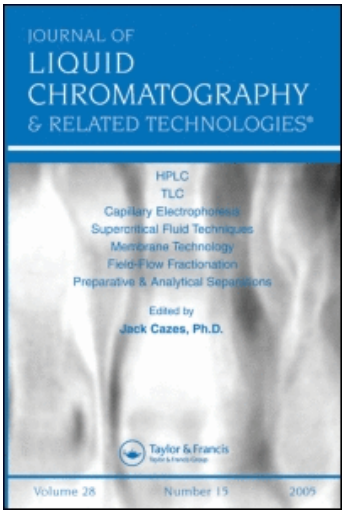

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S. Rompotis[a]; M. Parissi-Poulou[a]; E. Gikas[a]; M. Kazanis[a]; A. Vavayannis[a]; I. Panderi[a]
[a] Division of Pharmaceutical Chemistry, School of Pharmacy, University of Athens, Athens, Greece

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DETERMINATION OF VALPROIC ACID IN HUMAN PLASMA BY HPLC WITH FLUORESCENCE DETECTION

S. Rompotis, M. Parissi-Poulou, E. Gikas, M. Kazanis, A. Vavayannis, and I. Panderi*

University of Athens, School of Pharmacy, Division of Pharmaceutical Chemistry, Panepistimiopolis-Zografou, 157 71 Athens, Greece

ABSTRACT

A simple and sensitive high-performance liquid chromatographic (HPLC) method has been developed for the determination of valproic acid in human plasma. The method is based on pre-column derivatization using a new fluorescent reagent, *N*-(7-methoxy-4-methyl-2-oxo-2H-6-chromenyl)-2-bromoacetamide. The internal standard for the assay procedure was cyclohexanecarboxylic acid. The optimum monitoring conditions and the stability of the derivatives were investigated. The derivatization reaction proceeds in acetone in the presence of potassium carbonate and the crown ether, 18-crown-6 at 30°C with a reaction time of 30 min. The resulting derivatives were separated under isocratic conditions (acetonitrile–water, 60 : 40, v/v) on a LiChrospher RP-18 column (125 × 4.0 mm, i.d. 5 μm) and were detected fluorimetrically at a wavelength of 435 nm with an excitation of 345 nm. All chromatographic experiments were carried out at a flow rate 1.0 mL/min

*Corresponding author. E-mail: ipanderi@pharm.uoa.gr

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at ambient temperature. The method was fully validated and applied for the determination of valproic acid over the concentration range of 6.0–150.0 mg/mL. The detection limit for valproic acid added to plasma sample was 0.13 μg/mL plasma (3 pg on column). The recovery of valproic acid averaged 100.4%. The accuracy of the assay ranged from −0.5% to 1.3%. The method was shown to be highly reproducible and it seems to be adequate for routine therapeutic drug monitoring.

INTRODUCTION

Valproic acid, 2-propylpentanoic acid, is a simple branched-chain carboxylic acid used widely in the management of epilepsy[1] and several types of seizures, as it has a broad spectrum of activity.[2,3] It is rapidly and completely absorbed after oral administration and peak plasma concentrations are observed in 1 to 4 hours after a single oral dose.[4] The therapeutic plasma concentration is believed to be from 50 to 150 μg/mL.[5] The metabolism of valproic acid occurs mainly in the liver,[6] while almost no unmetabolized parent drug is excreted in the urine. In order to obtain information for therapeutic monitoring and optimize the clinical treatment of epilepsy, the development of a practical, reliable, and rapid analytical method for the determination of valproic acid in biological fluids is of great importance.

To follow the pharmacokinetics of valproic acid, several analytical methods have been reported in literature.[7] Most of these methods have been based on immunological methods[8] and gas chromatographic procedures with FID[9–12] or MS[13–17] detection. The high-performance liquid chromatographic (HPLC) analysis of valproic acid with direct detection[18,19] often presents problems because of the poor detectability due to the absence of a strong chromophore or fluorophore. High-performance liquid chromatography with UV or fluorescence detection, in combination with pre- or post-column chemical derivatization, constitutes a convenient approach to overcome this problem.[20] For the determination of valproic acid in biological fluids, various fluorogenic labeling reagents have been described, such as 4-bromophenacyl bromide,[21] 4-bromomethyl-7-methoxycoumarin,[22–25] and 6,7- methylenedioxy-1-methyl-2-oxo-1,2-dihydroquinoxaline-3-ylpropiono-hydrazide.[26]

Recently, *N*-(4-bromomethyl-7-hydroxy-2-oxo-2H-6-chromenyl) bromoacetamide (Br-MAMC) has been proposed as a useful fluorescent reagent for labeling carboxylic acids in the HPLC analysis.[27]

In the present study, the applicability of Br-MAMC to the HPLC analysis of valproic acid was evaluated, in view of the need of selective and sensitive methods to monitor the plasma levels of valproic acid. Cyclohexanecarboxylic

acid, which does not occur in biological fluids, was used as an internal standard for the precise determination. The precolumn derivatization procedure in human plasma samples was performed after the addition of acetonitrile for protein precipitation. Thus, no extraction or solvent evaporation steps are required. Optimum monitoring conditions and stability of the derivatives were investigated. The proposed method yielded accurate, rapid, and reproducible results for plasma samples spiked with valproic acid.

EXPERIMENTAL

Materials and Reagents

Solvents were of HPLC grade and were purchased from Lab-Scan Science Ltd., Ireland. Potassium carbonate and cyclohexanecarboxylic acid were obtained from Merck (Darmstadt, Germany), 1,4,7,10,13,16-hexaoxacycloctadecane,18-crown-6 ether was purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Water was deionised and further purified by means of a Milli-Q Plus Water Purification System, Millipore Ltd. Valproic acid of pharmaceutical purity grade was kindly provided by Sanofi Winthrop Industrie and used without any further purification.

Apparatus

The HPLC system was comprised of a Spectra-Physics SP8810 pump and a Perkin-Elmer LS30 spectrofluorometer, operated at an emission wavelength of 435 nm with an excitation of 345 nm. The chromatographic data were processed on a Spectra-Physics SP4270 integrator. Manual injections were carried out using a Rheodyne 7125 injector with a 20 μL loop. A heating block module, Silli-Therm, Pierce Chemical Company, equipped with thermostatically controlled heating (25–100°C) was used to perform the derivatization reaction.

Chromatographic Conditions

Chromatographic separations were performed on a LiChrospher 100, RP-18 stainless steel column (125 × 4.6 mm i.d., 5 μm particle size) Merck (Darmstadt, Germany), preceded by a BDS C-18, guard column (10 × 4.6 mm i.d., 5 μm), under isocratic conditions. The mobile phase, acetonitrile and water (60 : 40, v/v), was filtered through a 0.45 μm Millipore filter and degassed under vacuum prior to use. The mobile phase was pumped at a flow rate 1.0 mL/min. All chromatographic

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experiments were conducted at ambient temperature. Initially, excitation and emission wavelengths were adjusted at 345 and 435 nm, respectively.

Stock Standard Solutions

Stock standard solutions of the reagent *N*-(4-bromomethyl-7-hydroxy-2-oxo-2H-6-chromenyl) bromoacetamide (Br-MAMC), 40.0 μg/mL, and of the 18-crown-6, 1.0 mg/mL, were prepared by dissolving appropriate amounts of the corresponding compounds in acetone. These solutions were stored in the dark under refrigeration, and were found to be stable for a period of at least three weeks. Stock standard solutions of valproic acid, 120.0 μg/mL, and cyclohexanecarboxylic acid, 25.0 μg/mL, were prepared by appropriate dilution of the compounds in acetone and were found to be stable for several weeks at 4°C.

A series of working standard solutions of valproic acid were prepared by the appropriate dilution of the above-mentioned stock standard solution in acetone, to reach concentration range of 1.50–37.50 μg/mL.

Sample Preparation and Derivatization Procedure

Appropriate aliquots, 100 μL, of the valproic acid working standard solutions were placed in 5 mL conical bottom centrifuge tubes containing 25 μL of human plasma and 100 μL of the internal standard cyclohexanecarboxylic acid (25.0 μg/mL). Consequently, 275 μL of acetonitrile were added to the tubes for protein precipitation. The samples were then vortex mixed thoroughly for 30 sec and centrifuged at 2890 *g* for 5 min. A 50-μL aliquot of the clear supernatant reacted with 50 μL of the Br-MAMC solution (40.0 μg/mL). The reaction occurred in the presence of 50 μL of 1.0 mg/mL 18-crown-6 solution and 2 mg of a fine suspension of K_2CO_3, into an amber-colored micro-reaction vessel (3.0 mL). Corresponding aliquots of acetone were also added to reach a volume of 1000 μL. The reaction mixture was allowed to stand for 30 min at 30°C. Consequently, 100 μL of each reaction mixture was drawn and diluted with 100 μL of the mobile phase; 20-mL aliquots were injected onto the HPLC system.

Calibration Procedure

Two calibration curves were constructed by assaying aqueous and plasma samples spiked with valproic acid. Thus, two series of calibration standards were prepared in triplicate by adding 100 μL of the appropriate working standard solutions of valproic acid to 25 μL aliquots of water or drug-free human plasma,

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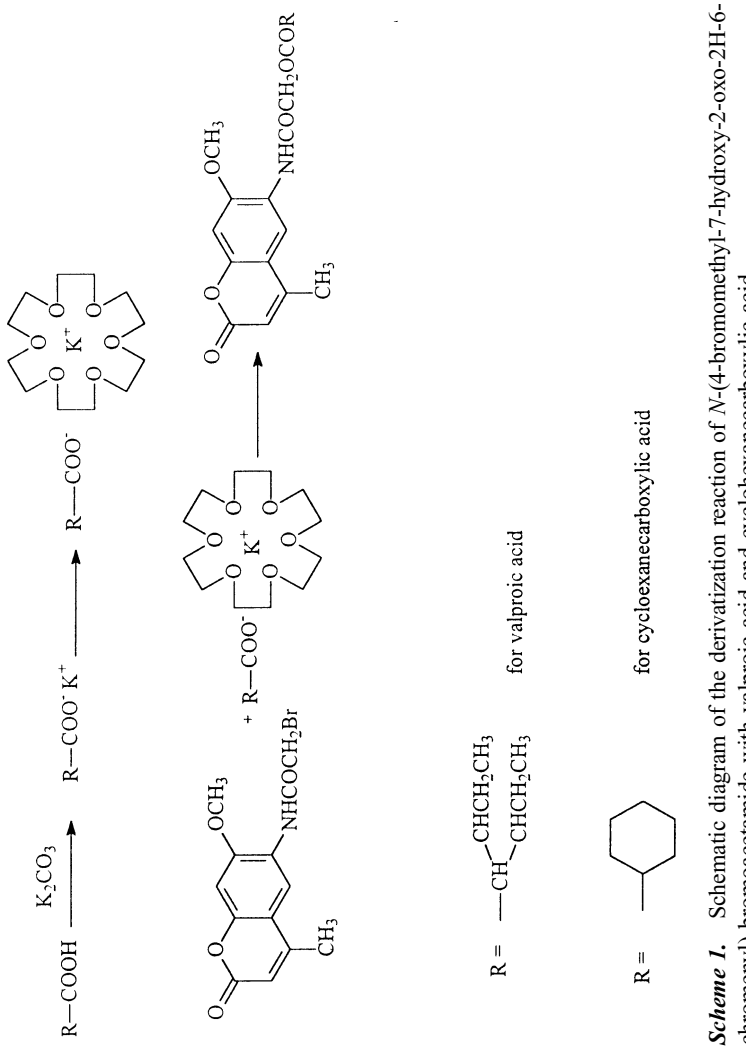

Scheme 1. Schematic diagram of the derivatization reaction of *N*-(4-bromomethyl-7-hydroxy-2-oxo-2H-6-chromenyl) bromoacetamide with valproic acid and cyclohexanecarboxylic acid.

respectively. The final concentrations of valproic acid in the spiked samples were: 6.0, 12.0, 24.0, 50.0, 72.0, 100.0, 120.0, and 150.0 μg/mL. Samples were analyzed immediately after their preparation, according to the above mentioned sample preparation and derivatization procedure. All calculations were based on peak area ratios. The chromatographic peak area of the ester derivative of valproic acid with Br-MAMC was divided by the peak area of the ester derivative of the internal standard with Br-MAMC. Concentrations of valproic acid in the analyzed samples were determined using the slopes and intercept values from a linear regression of the peak area ratios, vs. the concentration values from the calibration standards. To improve the accuracy of the assay throughout the lower calibration range, the regressions were weighted $1/\text{ratio}^2$.

RESULTS AND DISCUSSION

The applicability of Br-MAMC as a fluorogenic derivatization reagent for the analysis of valproic acid and cyclohexanecarboxylic acid (internal standard) was thoroughly investigated.

Optimization of the Derivatization Procedure

To achieve optimum conditions, the effects of the reaction time, temperature, concentration of Br-MAMC, solvent, and presence or absence of catalyst were investigated. In every step of the optimization reaction, all the contributing factors but one remain constant and the optimized value is used for the next experiment.

Fluorescence intensity was assessed initially for 30 min at 25, 30, 35, 40, 45, 50, and 60°C. The maximum reaction yield was achieved at 30°C. Lower temperatures were inadequate, whereas at temperatures higher than 50°C, the signal degraded slowly, perhaps through thermal decomposition of the product (Fig. 1). Consequently, the time profile of the reaction yield was monitored for 100 min, keeping the optimized temperature value of the reaction. The ester formation was completed and reached the maximum reaction yield within 30 min, while further heating did not increase the reaction yield (Fig. 2). The stoichiometric ratio between the reagent and valproic acid was also examined, and it was found that the reaction yield reached a plateau for ratios greater than 1 : 31, M/M (Fig. 3). Finally, the contribution of the catalyst, namely, the 18-crown-6 ether, was evaluated. The role of 18-crown-6 ether is to solvate K^+ in its polar cavity formed by the 6 oxygen atoms, leaving the anion of the acid countered by the opposite charge and, thus, more nucleophilic. The derivatization reaction of both of the acids with Br-MAMC proceeds much slower in the absence of 18-crown-6 ether (Fig. 4). The ester

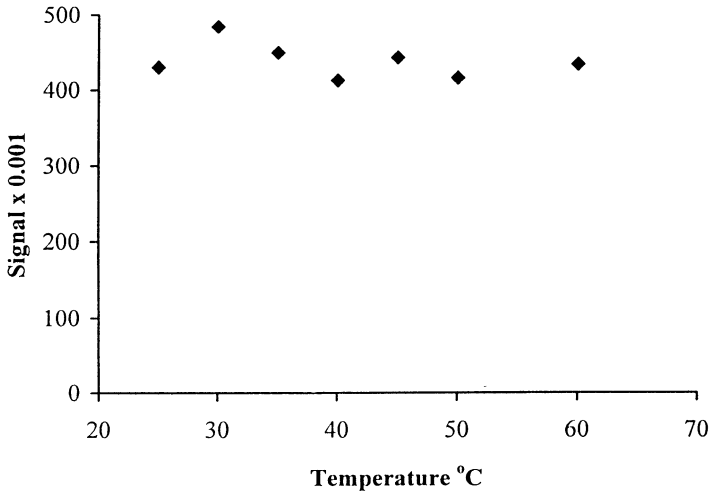

Figure 1. Influence of temperature on the derivatization of valproic acid with Br-MAMC; reaction time: 30 min; valproic acid–Br-MAMC: 1/31 M/M; in the presence of 18-crown-6 ether.

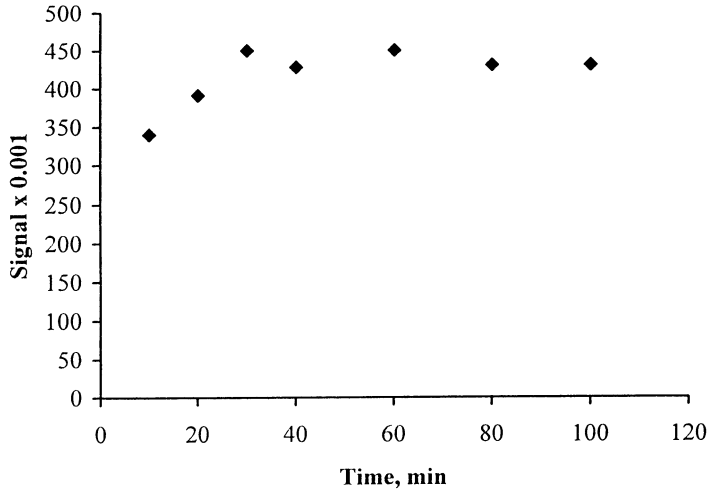

Figure 2. Influence of the reaction time on the derivatization procedure of valproic acid with Br-MAMC; temperature: 30°C; valproic acid–Br-MAMC: 1/31 M/M; in the presence of 18-crown-6 ether.

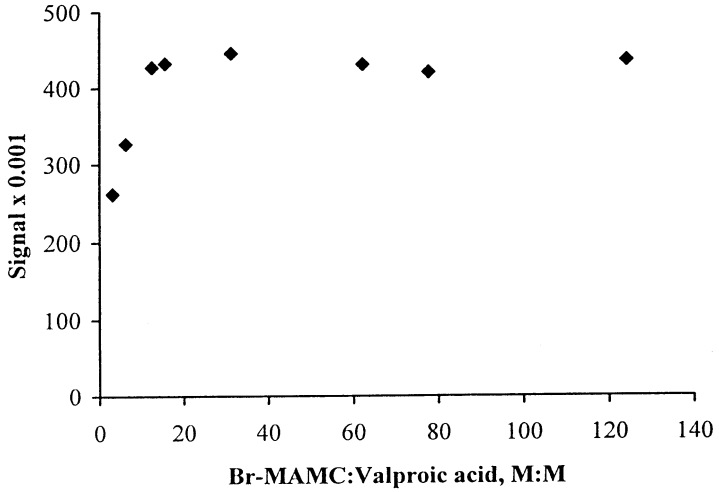

Figure 3. Influence of stoicheiometric ratio of valproic acid–Br-MAMC on the derivatization of valproic acid with Br-MAMC; reaction time: 30 min; temperature: 30°C; in the presence of 18-crown-6 ether.

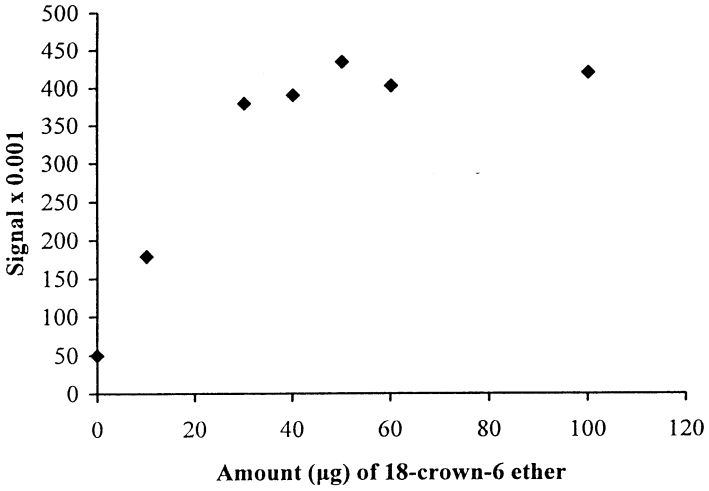

Figure 4. Influence of amount (µg) of 18-crown-6 ether on the derivatization of valproic acid with Br-MAMC reaction time: 30 min; temperature: 30°C; valproic acid–Br-MAMC: 1/31 M/M.

formation reached the maximum reaction yield in the presence of 50 μg of catalyst, whereas, further increase in the amount of the catalyst did not cause any increase in the reaction yield. The optimum conditions were the followings: temperature 30°C, reaction time: 30 min, ratio of valproic acid/Br-MAMC: 1 : 31, M/M, amount of 18-crown-6 ether: 50 μg. The stability of the Br-MAMC esters has also been investigated by keeping the reaction mixture in an ice-bath for 2 hr, prior to the HPLC analysis. As it can be seen in Fig. 5, no significant variations occurred in the signals upon periodic injections of the solution into the HPLC system.

Chromatographic Characteristics

Chromatographic separations were carried out on a Lichrospher 100, RP-18 column. The effect of composition of the mobile phase on the resolution of the esters was investigated; methanol and acetonitrile were tried as organic modifiers in the mobile phase in combination with water. The fluorescence intensity ($\lambda_{ex} = 345$ nm; $\lambda_{em} = 435$ nm) was found to be dependent on the nature of the organic modifier and decreased on increasing the modifier concentration in the mobile phase. Thus, for the valproic acid ester with Br-MAMC, the relative fluorescence intensities were as follows: $I = 100$ in methanol–water 50 : 50, v/v; $I = 89$ in methanol–water 75 : 25, v/v; I = 80 in acetonitrile–water 50 : 50, v/v; and $I = 65$ in acetonitrile–water 75 : 25, v/v. In the present study, acetonitrile was preferred to methanol as it gave better peak resolution. The best separation of the Br-MAMC derivatives of valproic acid and cyclohexanecarboxylic acid was achieved with a mobile phase of acetonitrile and water (60 : 40, v/v), which was pumped at a flow rate of 1.0 mL/min. Figure 6 shows

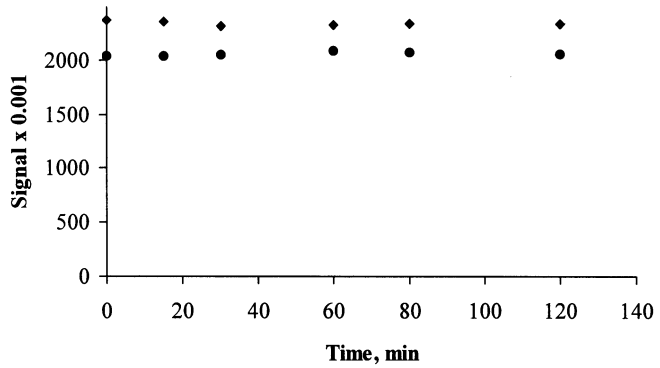

Figure 5. Stability of the signals of the esters of valproic acid (◆) and cyclohexanecarboxylic acid (●) with Br-MAMC.

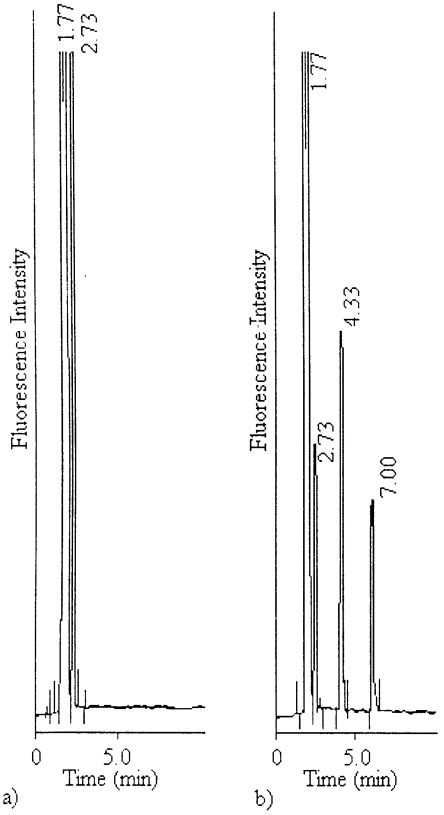

Figure 6. (a) Representative chromatogram of Br-MAMC alone; (b) Chromatographic separation of the excess of Br-MAMC and the esters of Br-MAMC with cyclohexanecarboxylic acid and valproic acid eluted at retention times 2.73, 4.33, and 7.00 min, respectively. Chromatographic conditions: reversed-phase HPLC on an Lichrospher-100 RP-18 column; mobile phase acetonitrile and water (60 : 40, v/v); flow rate 1.0 mL/min, fluorescence detection with $\lambda_{ex} = 345$ nm and $\lambda_{em} = 435$ nm.

a typical chromatogram obtained from the analysis of plasma samples spiked with valproic acid and cyclohexanecarboxylic acid and derivatized with Br-MAMC. The separation of the excess of Br-MAMC and the esters of Br-MAMC with valproic acid and CLC was completed within 8 min. Both of the esters of cyclohexanecarboxylic acid and valproic acid with Br-MAMC are baseline separated from each other, with retention times of 4.33 and 7.00 min, respectively.

Statistical Analysis of Data

Under the chromatographic conditions described in the experimental section, linear relationship was observed between the ratio of the peak area of valproic acid to that of the internal standard and the corresponding concentration of valproic acid, as described in Table 1. The correlation coefficient (r) and the standard error of the estimate (S_r) of the calibration lines are also given, along with the standard deviation (S.D.) of the slope and the intercept. In order to further evaluate the linearity of the proposed method, five calibration equations were constructed by assaying plasma samples spiked with valproic acid over a period of four weeks. The average regression equation is:

$$R = 0.0106(\pm 2.0 \times 10^{-4}) \times C_{\text{val}} - 0.0433(\pm 3.0 \times 10^{-3})$$

where C_{val} is the concentration of valproic acid. The correlation coefficient invariably exceeded 0.9993.

The limit of detection, D_L, and the limit of quantitation, Q_L were attained, as defined by IUPAC[28]and ICH Topic Q2B,[29] using the slope (b) of the calibration graph and the standard deviation, S_α, of the intercept (α). Thus, another calibration curve was constructed by assaying plasma samples spiked with valproic acid over the concentration range 3.0 to 6.0 μg/mL. Linear regression analysis of data gave the following equation:

$$R = 0.0060(\pm 5.1 \times 10^{-5}) \times C_{\text{val}} - 0.0173(\pm 2.3 \times 10^{-4})$$
$$r = 0.9998; S_r = 1.3 \times 10^{-4}$$

Table 1. Analytical Data of the Calibration Graphs Obtained by the HPLC-Fluorescence Method for Valproic Acid Derivatized with Br-MAMC

Fluid	Linearity Range (μg/mL)	Calibration Equation[a]	r[b]	S_r[c]
Aqueous solution	6.0–150.0	$R = 0.0094(\pm 4.7 \times 10^{-5}) \times C_{\text{val}} - 0.0413\ (\pm 4.0 \times 10^{-4})$	0.99998	0.012
Plasma	6.0–150.0	$R = 0.0108(\pm 1.7 \times 10^{-4}) \times C_{\text{val}} - 0.0464\ (\pm 1.3 \times 10^{-3})$	0.9998	0.028

[a]Ratio of the peak area amplitude of the valproic acid ester with Br-MAMC to that of the internal standard ester with Br-MAMC, R, vs. concentration of valproic acid, C_{val}, in μg/mL; eight standards.
[b]Correlation coefficient.
[c]Standard error of the estimate.

where R is the ratio of the peak area amplitude of the valproic acid ester with Br-MAMC to that of the internal standard ester with Br-MAMC, and C_{val} the concentration of valproic acid. The limit of detection, calculated from $y - \alpha = 3.3 \times S_\alpha$ and $y - \alpha = b \times D_L$ was found to be 0.1 μg/mL in plasma (3 pg on column). The limit of quantitation, calculated from $y - \alpha = 10 \times S_\alpha$ and $y - \alpha = b \times D_L$ was found to be 0.38 μg/mL in plasma.

Intra-day precision and accuracy of the method were assessed by analyzing spiked plasma samples at low (6.0 μg/mL), middle (72.0 μg/mL) and high (150.0 μg/mL) concentrations. Five replicates of each concentration were analyzed and results are given in Table 2. To evaluate precision, the mean values and the RSD% values were calculated for each concentration. The intra-day precision was found to be acceptable, with the RSD.% values ranging from 0.38 to 0.10. Moreover, the accuracy was assessed by calculating the relative percentage error (E_r%), which ranged from −0.3 to 1.0.

The inter-day data for the precision and accuracy were also determined by analyzing five sample batches of spiked plasma samples at low (6.0 μg/mL), middle (72.0 μg/mL), and high (150.0 μg/mL) concentrations on five separate days. The inter-day RSD and E_r% values ranged from 0.40 to 0.03 and −0.5 to 1.3, respectively. Results are also presented in Table 2.

The results of the recovery of the method from plasma samples spiked with valproic acid at theoretical concentrations 6.0, 72.0, 150.0 μg/mL are shown on

Table 2. Precision and Accuracy of Within- and Between-Run Analysis for the Determination of Valproic Acid by HPLC

	Assayed Concentration of Valproic Acid (μg/mL)		
Nominal Concentration (μg/mL)	Mean ± S.D.	RSD (%)[a]	E_r (%)[b]
Intra-day ($n = 5$)			
6.0	5.98 ± 0.02	0.33	−0.3
72.0	71.84 ± 0.28	0.39	−0.2
150.0	151.50 ± 0.20	0.13	1.0
Inter-day ($n = 5$)			
6.0	5.97 ± 0.02	0.33	−0.5
72.0	72.40 ± 0.12	0.16	0.6
150.0	151.90 ± 0.05	0.03	1.3

[a]Percentage relative standard deviation.
[b]Relative percentage error = [(assayed concentration − nominal concentration)/nominal concentration] × 100.

Table 3. Percentage Recovery of Valproic Acid in Plasma

Theoretical Concentration in Plasma (μg/mL)	Measured Concentration in Plasma (μg/mL)	n[a]	Mean Recovery ± S.D.
6.0	5.97	10	99.4 ± 0.4
72.0	72.36	10	100.1 ± 0.1
150.0	151.80	10	101.2 ± 0.1

[a]n = number of measurements.

Table 3. The analytical recovery of valproic acid in concentration 6.0, 72.0, and 150.0 μg/mL are 99.5, 100.1, and 101.3, respectively.

A number of drugs were tested for possible interference in the assay of valproic acid. The results presented in Table 4 shows that the selected drugs cause no interference.

CONCLUSIONS

In conclusion, *N*-(4-bromomethyl-7-hydroxy-2-oxo-2H-6-chromenyl) bromoacetamide is a useful fluorogenic labeling reagent suitable for the prechromatographic derivatization of valproic acid. It provides a simple solution for the problem of low absorptivity of valproic acid and permits the determination of valproic acid in a small amount of human plasma without any extraction

Table 4. Possible Interference to the Assay of Valproic Acid

Co-administered Drugs		Assayed Concentration of Valproic Acid (μg/mL)[b]		
Drug Added	C (μg/mL)[a]	Mean ± S.D. (n = 3)	S_r%[c]	E_r%[d]
Phenytoin	0.5	72.42 ± 0.34	0.47	0.6
Primidone	5.0	72.12 ± 0.22	0.31	0.2
Carbamazepine	8.0	72.36 ± 0.32	0.44	0.5
Phenobarbital	3.0	72.34 ± 0.21	0.29	0.5

[a]Drug concentrations are mean plasma concentrations.
[b]Valproic acid concentration added was 72.00 μg/mL.
[c]Relative standard deviation.
[d]Relative standard error.

procedure. Thus, the technique is suitable for volatile valproic acid and offers higher sensitivity to permit the determination of this drug in 25 μL plasma samples. Therefore, this method can be considered real interest for the rapid and reliable clinical and pharmacokinetic studies of the valproic acid.

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