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Short Communication

Determination of valproic acid by high-performance liquid chromatography with photodiode-array and fluorescence detection

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ABSTRACT

The derivatization of valproic acid and undecylenic acid with 4-bromomethyl-7-methoxycoumarin is described. The derivatives were detected by photodiode-array and fluorescence detectors. The optimum monitoring conditions and the stability of the suspension solution and derivatives were investigated. A high-performance liquid chromatographic (HPLC) method with isocratic or gradient elution has been established for the analysis. This method has been used for the determination of total and free valproic acid in serum. There is a satisfactory correlation between the results obtained by this HPLC method and those measured by the enzyme immunoassay. Some other common anti-epileptic drugs did not interfere with the analysis. The method is simple and fast and has better sensitivity and linearity than enzyme immunoassay. It is suited for routine therapeutic drug monitoring.

INTRODUCTION

Valproic acid (VPA) is a commonly used anti-epileptic drug because of its wide spectrum of activity and low central nervous system toxicity [1]. Monitoring VPA levels in serum of epileptic patients is desirable mainly for adjusting dosage, avoiding side-effects and assessing patient compliance [1,2]. Usually, VPA is determined by gas chromatography–mass spectrometry (GC–MS) or immunoassay. GC–MS is suitable for the simultaneous determination of VPA and its metabolites [2,3]. The disadvantages of GC-based methods commonly are involved, lengthy and cumbersome multiple-step procedures for sample preparation, such as solvent extraction, evaporation and derivatization [3,4]. These procedures may also result in loss of sample and poor reproducibility. Immunoassays are unable to determine VPA and its metabolites simultaneously. Another disadvantage is that the concentration of VPA shows a hyperbolic rather than a linear relationship to the change in absorbance in the enzyme immunoassay.

Only a few high-performance liquid chromatographic (HPLC) methods have been reported for the analysis of VPA because VPA lacks nitrogen and a ring moiety and therefore has no chromophoric characteristics [5]. The volatility of VPA causes an extra problem during the solvent evaporation step required by derivatization in its determination. Many fluorescence-labelling re-

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agents have been described for the derivatization of carboxylic acids. Among them 4-bromomethyl-7-methoxycoumarin (BrMMC) may be the most suitable, because of its small molecular size [6]. This is a sensitive fluorophore and UV-labelling reagent and the resulting esters are easily eluted on a reversed-phase column with water–alcohol mixtures without addition of salts or counter ions [7]. Esterification with BrMMC has been applied to fatty acids [8–10], dicarboxylic acids [11], prostaglandins [12], 5-fluorouracil and 1,5-fluorouracil [13,14], pyrimidine compounds [15] and glucuronic acid conjugates [7,16]. Recently, VPA has been treated with BrMMC to form a fluorescent derivative [17,18]. The derivatization is performed after acetonitrile is added to the serum. No extraction or solvent evaporation steps are required [18].

The present paper describes the use of photodiode-array and fluorescence detectors for the detection of the BrMMC derivative of VPA. Optimum monitoring conditions and stability of the derivatives were investigated. An HPLC method with isocratic or gradient elution has been established for the analysis. This method has been used for the determination of total (protein-bound) and free (unbound) VPA concentrations in serum. The results show a good correlation with the levels obtained by the enzyme immunoassay method. Other common anti-epileptic drugs do not interfere with the determination.

EXPERIMENTAL

Apparatus

Our HPLC system was a Hewlett-Packard HP 1090 M, including a DR 5 solvent delivery system with three channels. Samples were injected by an autoinjector and autosampler. The column effluent was monitored with an HP 1040A photodiode-array UV detector and an HP 1046 programmable fluorescence detector in series. Data were collected and analyzed on an HP 79994A analytical workstation. The column was an HP Hypersil-ODS 5-μm column (100 mm × 2.1 mm I.D.).

Reagents and chemicals

Acetonitrile, methanol and distilled water were HPLC grade from Curtin Matheson Scientific (Houston, TX, USA). 2-Propylpentanoic acid (VPA), undecylenic acid (UDA), 18-crown-6, potassium carbonate and BrMMC were all analytical-grade reagents purchased from Sigma (St. Louis, MO, USA). These reagents were used without further purification.

Derivatization procedures

The 18-crown-6 suspension solution was prepared by dissolving 100 mg of potassium carbonate in 50 μl of water; this solvent was then added to 5 ml of 20 m*M* 18-crown-6 in acetonitrile. After mixing in an ultrasonic bath for 30 min, another 5 ml of acetonitrile were added [19]. The derivatization was performed as follows: to 25 μl of standard solution, serum sample or its ultrafiltrate, 475 μl of acetonitrile containing the internal standard (10 μg/ml UDA in acetonitrile) were added. After centrifugation, 50 μl of the supernatant were added to an autosampler vial containing 100 μl of suspension solution and 50 μl of BrMMC solution (1 mg/ml BrMMC in acetonitrile). After reaction at 65°C in the dark for 30 min, 5 μl were injected into the HPLC system.

Sample preparation

Blood samples were collected from patients monitored for therapeutic drugs in the clinical laboratory of our hospital. The VPA concentration of each sample was assayed with Emit SYVA enzyme immunoassay reagents (SYVA, Palo Alto, CA, USA) on the Monarch 2000 Chemistry System (Instrumentation Laboratory, Lexington, MA, USA). The ultrafiltrate was prepared with a Centrifree unit (Amicon, Beverly, MA, USA). The serum sample (200 μl) was put in the Centrifree unit and centrifuged for 10 min at 700 *g*. About 50 μl of ultrafiltrate were collected for analysis.

Chromatographic conditions

HPLC for VPA was performed with either isocratic or gradient elution at a flow-rate of 0.3 ml/min. The column temperature was kept at 40°C.

For isocratic elution, the mobile phase was methanol–water (80:20, v/v). For gradient elution, solvent A was methanol–water (60:40, v/v) and solvent B was pure methanol. The time table for gradient elution was: (1) 100% solvent A for 3 min, (2) from 3 to 8 min a linear increase of solvent B to 100%; (3) from 8 to 11 min a return to 100% solvent A; (4) a hold at 100% solvent A from 11 to 15 min. The derivatives were detected with a fluorescence detector at an excitation wavelength (Ex) of 322 nm and an emission wavelength (Em) of 695 nm, and with the photodiode-array detector at sample wavelengths of 322 and 200 nm and bandwidths of 4 and 10 nm, respectively, with 500-nm reference wavelength and 100-nm reference bandwidth.

RESULTS AND DISCUSSION

Derivatization and detection

A photodiode-array detector was used for providing more information by multi-signal detection, checking peak purity and obtaining better baseline for gradient elution by using the reference wavelength. The UV spectra of VPA obtained from on-line detection are shown in Fig. 1. The BrMMC derivative of UDA had UV spectra similar to those of VPA, with the strongest peak at 200 nm and two other peaks at 322 and 220 nm. Detection of VPA and UDA at 200 nm was about three times more sensitive than that at 322 nm, but it was compromised by the potential interference at low-UV wavelength. At 322 nm, the sensitivity was less than at 200 and 220 nm, but there was also less chance of interference. Both 322 and 200 nm were selected as sample wavelengths in this experiment. The 322-nm sample wavelength was used for its selectivity and 200 nm was used for its sensitivity.

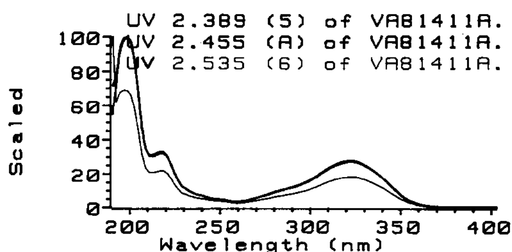

Fig. 1. UV spectra of VPA, plotted from the signals of the photodiode-array detector.

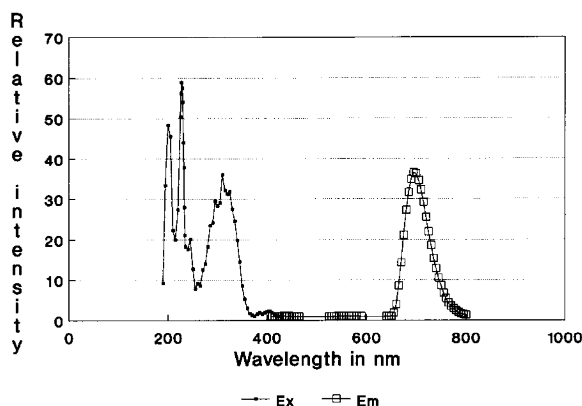

Fig. 2. Fluorescence spectra of VPA, obtained from stopped-flow scanning.

Most references report that Ex 322 nm and Em 435 nm were used for the fluorescence detection of BrMMC derivatives [9–13,15]. When we performed stopped-flow scanning, the Ex was 322 nm which was close to the reference value. Although changing the Ex from 322 to 228 nm could improve the sensitivity by 1.57 times, the longer and more selective Ex of 322 nm was used to avoid interference at low wavelength due to other substances which might be present in physiological fluids. During the Em scanning, although there was a peak around 435 nm, another much stronger fluorescence peak appeared at 695 nm. Setting the Em at 695 nm for the analysis resulted in a stable and reproducible signal with significantly enhanced sensitivity and excellent linearity. So the Em was set at 695 nm for the quantitation in this experiment. The fluorescence spectra of VPA obtained by stopped-flow scanning are shown in Fig. 2.

Chromatography

With both gradient and isocratic elution a satisfactory separation was achieved. (Figs. 3 and 4). The reproducibility of the retention time with gradient and isocratic elution was investigated. All of these determinations were made in ten consecutive analyses. For gradient elution, the coeffi-

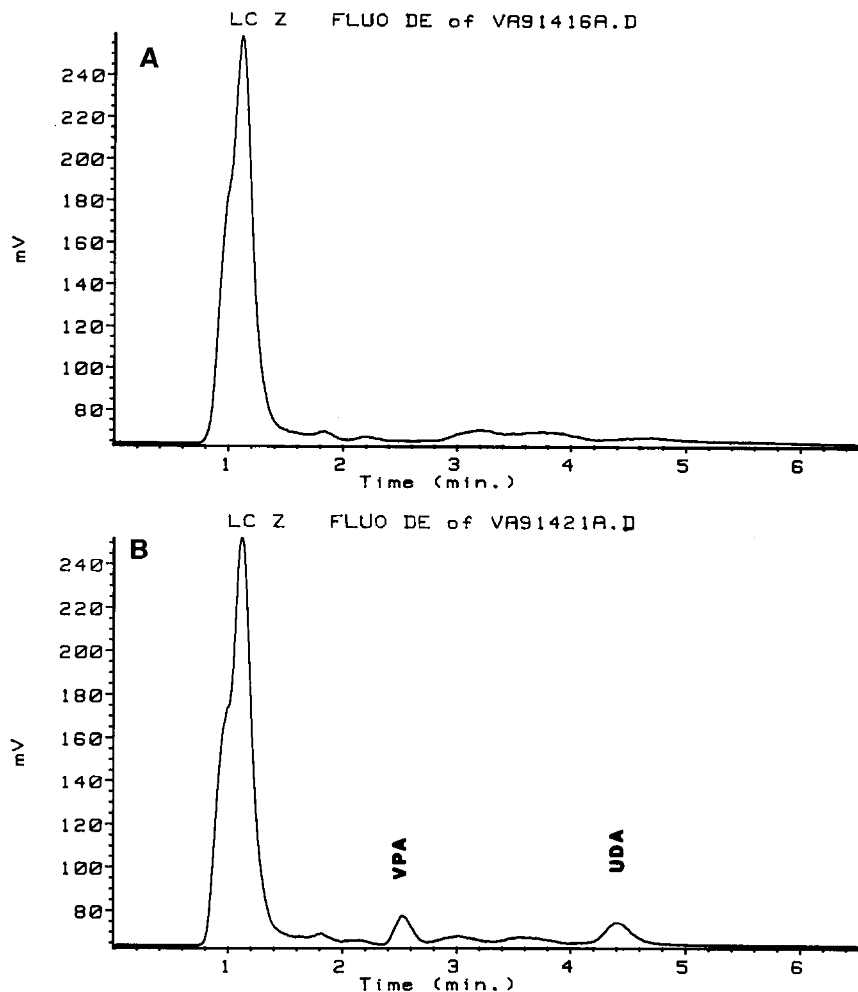

Fig. 3. Isocratic HPLC of (A) a blank serum sample and (B) a patient's serum sample spiked with UDA. See Experimental for chromatographic conditions. The signal was detected by a fluorescence detector.

cient of variation of the retention time was 0.001 and 0.002 for VPA and UDA, respectively. For isocratic elution, the coefficient of variation was 0.002 and 0.003. Ambient temperature was used by other investigators for the analysis of VPA derivatives [18]. We found that increasing the column temperature to 40°C reduced the retention time and lowered the column pressure. The constant column temperature was also a key factor for the stability of the retention time. The reproducibility of peak areas for VPA and UDA in isocratic and gradient elution were tested by five analyses. The coefficients of variation were within 5% for both within- and between-day analysis. The isocratic elution procedure was fast, simple and suitable for routine analysis, but it was probably not suitable for the analysis of the metabolites of VPA since the retention time of VPA was too close to t_0, leaving no room for the metabolites. Gradient elution is more complex but has potential for better separation of the metabolites of VPA.

We did not observe a memory effect due to the presence of unreacted fatty acids on the top of the column [18] or interference of unreacted BrMMC [15].

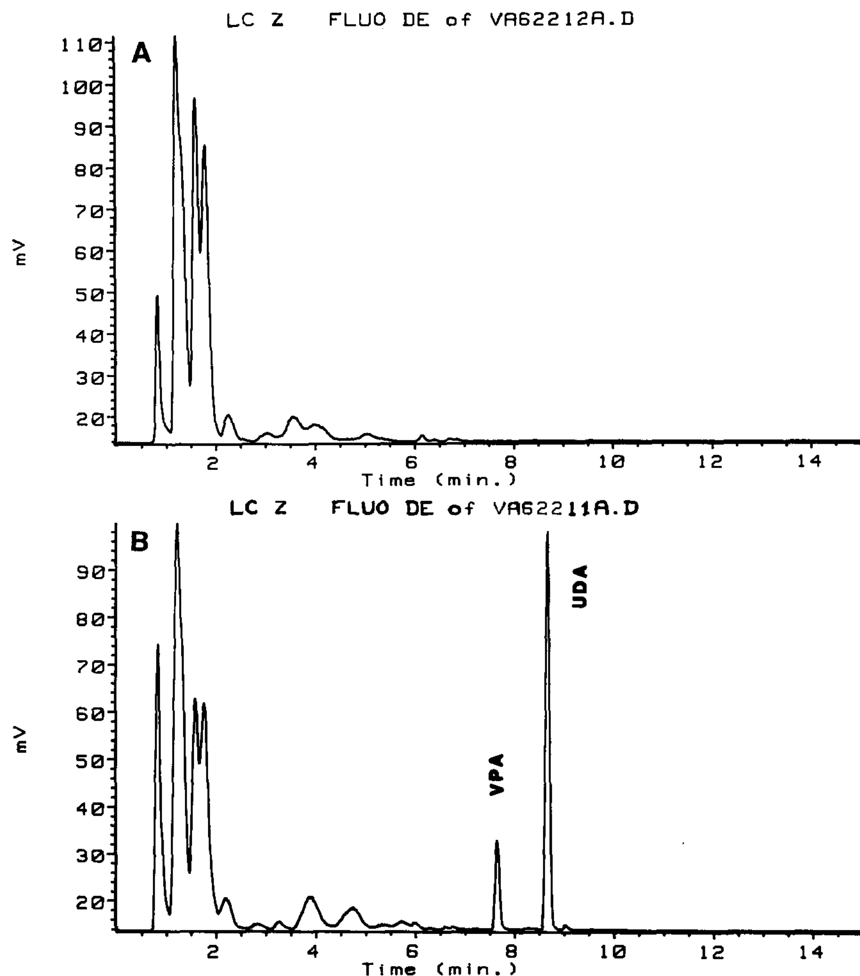

Fig. 4. Gradient HPLC of (A) a blank serum sample and (B) a patient's serum sample spiked with UDA. See Experimental for chromatographic conditions. The signal was detected by a fluorescence detector.

Stability of the derivatives

The stability of the BrMMC glucuronide esters has been determined by keeping the reaction mixture in ice for 2 h. After this time, no significant variations occurred upon periodic injections of the solution into the chromatograph [7]. The BrMMC esters have also been reported to be stable in boiling solvents [8]. To investigate the stability of VPA and UDA derivatives, several vials with identical concentrations of VPA and UDA were prepared and derivatized under the same conditions, except that the incubation periods were varied (15, 30, 45 min, 1, 2, 4 and 8 h) at 65°C. The reaction solutions were analyzed by HPLC and peak areas were compared at the various time intervals. The results are shown in Fig. 5. The derivatives were relatively stable using UV detection even at the high temperature of 65°C for 8 h. The peak areas of VPA and UDA decreased by only 8% during a period of 8 h whereas the fluorescence intensity diminished by about 18% during the same period. It should be mentioned that the fluorescence ratio of VPA to UDA was constant at all points of the 8 h period. The variance was less than 5%. These results indicate that both internal standard and external standard methods can be used for UV quantitation. The internal standard method is recom-

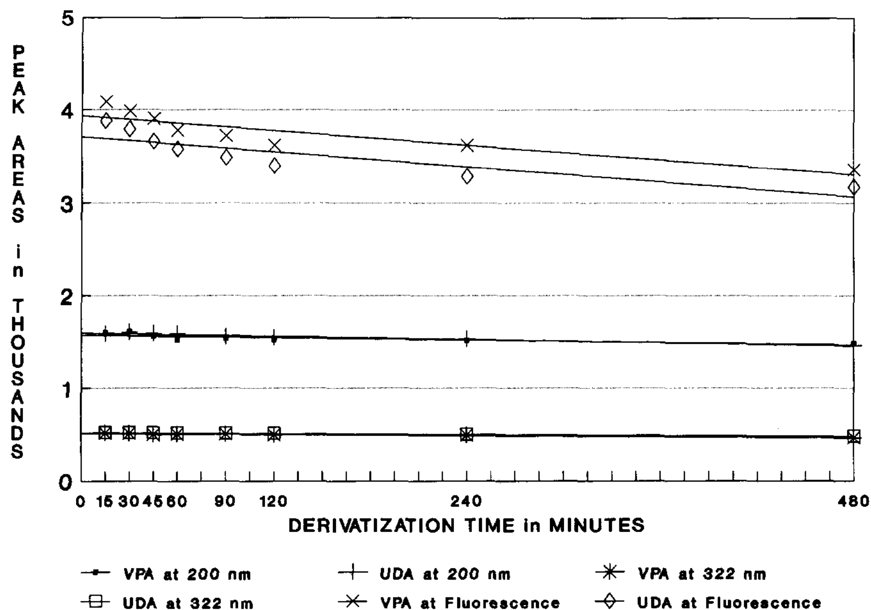

Fig. 5. Stability of VPA and UDA derivatives at the reaction temperature of 65°C.

mended for fluorescence detection. Since the fluorescence intensity was not as stable as UV absorbance whereas the fluorescence ratio of VPA to UDA was constant, better quantitation can be expected by using the internal standard method.

The BrMMC derivatives slowly hydrolyze in the presence of water [11]. It has been mentioned that the water sensitivity of the fluorescence is likely to cause inconvenience when comparisons are made between reversed-phase isocratic separation differing in solvent composition and solvent-programmed separation [10]. We investigated this influence by comparison of the results obtained from the same sample eluted by isocratic and gradient elution (Table I). Another sample was analyzed by the use of two different gradient mobile phase systems. Methanol or acetonitrile was used as organic solvent in these two mobile phase systems. The influence of mobile phase on the peak area of VPA and UDA is shown in Table II. Although there was no significant difference in the peak areas of UV signals between the gradient and isocratic elution and the two mobile phase systems, the relative fluorescence intensity was stronger with isocratic elution and with the mobile phase system using methanol as organic solvent. The reason for this is not known.

TABLE I

INFLUENCE OF GRADIENT AND ISOCRATIC ELUTION ON PEAK AREAS

Compound	Detection	Peak area	
		Gradient elution	Isocratic elution
VPA	UV, 322 nm	94.45	96.48
UDA	UV, 322 nm	70.69	71.32
VPA	UV, 200 nm	330.34	330.66
UDA	UV, 200 nm	235.69	241.26
VPA	Fluorescence	554.99	640.19
UDA	Fluorescence	283.74	463.20

The stability of the suspension solution was also investigated. The same sample was derivatized by three suspension solutions prepared 1, 14 and 28 days apart, and was analyzed under identical chromatographic conditions. The peak areas of VPA and UDA were very similar in these three suspension solutions, indicating that the suspension solution was stable for at least 28 days.

TABLE II

INFLUENCE OF MOBILE PHASE ON PEAK AREAS

Compound	Detection	Peak area	
		Mobile phase I[a]	Mobile phase II[b]
VPA	UV, 322 nm	39.16	38.18
UDA	UV, 322 nm	222.26	215.07
VPA	UV, 200 nm	114.45	116.71
UDA	UV, 200 nm	634.77	624.92
VPA	Fluorescence	258.09	117.95
UDA	Fluorescence	1055.0	483.26

[a] Mobile phase I: (A) methanol–water (40:60, v/v); (B) methanol.

[b] Mobile phase II: (A) Acetonitrile–water (50:50, v/v); (B) acetonitrile.

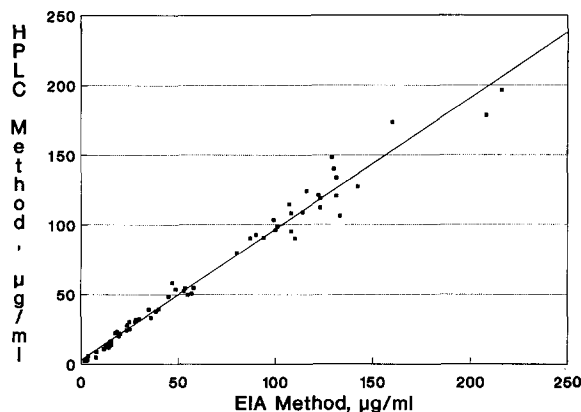

Fig. 6. Correlation between the VPA levels of 65 serum samples detected by enzyme immunoassay and HPLC (y = 2.786 + 0.940x, r^2 = 0.978).

Application

The calibration plot of VPA was linear from 6.25 to 200 µg/ml determined by both UV and fluorescence detectors. The correlation coefficients were 0.9993, 0.9993 and 0.9995 for UV at 200 nm, UV at 322 nm and fluorescence, respectively. This covered the therapeutic range of VPA. The same amounts of VPA and UDA were added to three drug-free serum samples. These samples were then derivatized and analyzed. The peak areas obtained were compared with the results obtained by the analysis of pure drug standard. The analytical recovery rates of VPA and UDA were near 95% for both VPA and UDA.

The levels of 35 total serum VPA and 30 free VPA determinations were compared with those obtained by enzyme immunoassay. The correlation between these two methods is satisfactory (Fig. 6). Some other drugs, particularly the other anti-epileptic drugs, may be co-administrated in some of the patients. Therefore several drugs were investigated by using the same derivatization and chromatographic conditions to see if these compounds would interfere with the detection of VPA. Phenobarbital, phenytoin and carbamazepine did not interfere within the therapeutic range of these drugs.

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