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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF VALPROIC ACID IN PLASMA USING A MICELLE-MEDIATED PRE-COLUMN DERIVATIZATION

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SUMMARY

A method for the determination of valproic acid (2-propylpentanoic acid) in plasma by high-performance liquid chromatography (HPLC) after pre-column derivatization is described. The derivatization of valproic acid with a fluorophore and UV label, 4-bromomethyl-7-methoxycoumarin, is performed in plasma diluted with an aqueous micellar system. No extraction or solvent evaporation steps are required. The mechanism of the derivatization of the carboxylic acid is based on phase-transfer catalysis. The sample preparation, including the derivatization step, is rapid and very simple. The proposed HPLC-method was evaluated and compared with a standard immunological assay used for the determination of valproic acid in plasma.

INTRODUCTION

Valproic acid (VPA) (2-propylpentanoic acid) is a commonly used anti-epileptic drug and its therapeutic level in plasma ranges from *ca.* 260 to 840 μM[1,2]. Usually VPA is monitored by gas chromatography (GC) or by an immunological assay[3]. GC analysis is hampered by the fact that it is necessary first to extract the drug with an organic solvent. VPA can be monitored directly using immunoassays. However, these techniques are expensive and it is not possible to determine several anti-epileptic drugs simultaneously, in contrast to, *e.g.*, high-performance liquid chromatography (HPLC)[4].

Only a few HPLC methods have been reported for the analysis of VPA[3,4]. Because of the low detectability, the quantification of VPA in plasma is often performed after the attachment of a suitable chromophore or fluorophore to the carboxylic function[3]. The problems of derivatization procedures prior to HPLC analysis are in general caused by the necessary extraction of the drug with a suitable organic solvent. The volatility of VPA during the solvent evaporation steps causes an extra problem in its determination[3]. In this paper we report an HPLC method for the

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determination of VPA in plasma after a simple labelling procedure with a sensitive fluorophore and UV label[5], 4-bromomethyl-7-methoxycoumarin (BrMMC). This labelling with BrMMC is performed in plasma after the addition of an aqueous micellar system[6]. The mechanism of the micelle-mediated derivatization of carboxylic acids is described in more detail in the accompanying paper[6].

EXPERIMENTAL

Chemicals and solutions

Millipore water (Milford, MA, U.S.A.) was used throughout. Except for Arkopal N-130, the chemicals were of analytical-reagent grade.

The non-ionic surfactant Arkopal N-130 (a condensate of nonylphenol and a polyoxyethylene chain of 13 units on average) was kindly supplied by Hoechst Holland (Amsterdam, The Netherlands). Tetrahexylammonium bromide (THxABr) and 4-bromomethyl-7-methoxycoumarin (BrMMC) were obtained from Fluka (Buchs, Switzerland). Acetonitrile, acetone (distilled before use), methanol, 18-crown-6 ether and nonanoic acid (NA) were obtained from Merck (Darmstadt, F.R.G.), 10-undecenoic acid (UA) from OPG (Utrecht, The Netherlands), valproic acid (VPA) from Sigma (St. Louis, MO, U.S.A.) and sodium valproate (Na-VP) from LaBaz (Brussels, Belgium). The Na-VP was dried at 105°C prior to use; the water content was less than 1%. A 50 mM phosphate buffer (pH 7.0) was used throughout.

Nonanoic acid, 10-undecenoic acid and valproic acid were dissolved in acetone and stored at 4°C. Sodium valproate was dissolved in water and stored at 4°C.

Preparation of incubation vials

A 1.65-g amount of Arkopal N-130, 650 mg of THxABr and 60 mg of BrMMC were dissolved in 20 ml of acetone. After the transfer of 240 μl of this solution into a type 3814 reaction vessel (Eppendorf, Hamburg, F.R.G.) the acetone was evaporated at room temperature under a stream of nitrogen. Because of the photosensitivity of the label, the preparation of the incubation vials was performed with exclusion of light. The prepared vials were stored at -20°C and used for the incubation method 2 (see below).

Incubation procedures

Method 1. The preliminary experiments were based on the incubation procedure described previously[6]. A micellar stock solution of 100 mM Arkopal N-130 and 72 mM THxABr was prepared in 50 mM phosphate buffer (pH 7.0). Of this micellar solution 250 μl were added to 250 μl of a mixture of plasma and buffer and 10 μl of an internal standard solution were added. The derivatization was started by the addition of 25 μl of an acetone solution of 8 mg/ml BrMMC. The samples were derivatized at 70°C in a water-bath.

Method 2. In the new derivatization procedure in general 400 μl of buffer and 100 μl of patient's or spiked plasma were placed in a prepared incubation vial and 10 μl of an internal standard were added. After vortexing for 5 s the vials were incubated at 70°C in a water-bath for *ca.* 40 min.

The derivatizations using methods 1 and 2, which were performed with the exclusion of light, were terminated by the addition of 500 μl of acetonitrile. The

plasma samples were centrifuged at 3000 g for 5 min after the addition of acetonitrile. Volumes of 20 μl of the samples were injected into the HPLC system.

In addition to the incubations in the aqueous micellar system derivatizations were also performed in acetone. The procedure for incubation in acetone was described previously[6,8]. The carboxylic acid was added to an acetone solution that contained 18-crown-6 ether and potassium carbonate. The reaction was started by the addition of BrMMC (25 μl of an 8 mg/ml solution in acetone). The incubation was performed for 30 min at 60°C.

Chromatographic system

Samples of 20 μl of the diluted incubation mixture were injected into a fully automated HPLC system consisting of a Chromspher C_{18} (5 μm) column (100 × 3.0 mm I.D.) (Chrompack, Middelburg, The Netherlands), a laboratory-made precolumn of LiChroprep RP-8 (5–20 μm) (10 × 3.00 mm I.D.) (Merck), two M 6000 A pumps and an automated gradient controller (all from Waters Assoc., Milford, MA, U.S.A.) controlled by a Promis autosampler (Spark, Emmen, The Netherlands).

After the initial step with methanol–water (80:20, v/v) for 3 min a linear gradient was run to 100% methanol in 6 min, the final conditions being maintained for 4 min. The solvents were deaerated ultrasonically prior to use.

A Model 650 fluorescence detector (Perkin-Elmer/Hitachi, Tokyo, Japan) was used. The optimized exitation and emission wavelengths were 330 and 395 nm, respectively. A Waters 440 absorbance detector was used for UV detection at 340 nm. Retention times and peak areas were measured with an SP 4270 integrator (Spectra-Physics, Santa Clara, CA, U.S.A.).

Patients' samples

The patients' samples were those submitted for therapeutic drug monitoring to the clinical laboratory of the University Hospital of Utrecht. The total VPA concentration of the patients' plasma had been determined with the use of a fluorescence polarization immunoassay (FPIA) with a TDx analyser (Abbott, Irving, TX, U.S.A.). After the FPIA analysis the samples were immediately analysed using the HPLC procedure or were stored at −20°C prior to analysis.

RESULTS AND DISCUSSION

The principles of the procedure for the derivatization of carboxylic acids in the presence of the micellar system are described in more detail in the accompanying paper[6]. It was suggested that the mechanism is based on phase-transfer catalysis (PTC)[7]. In micellar PTC (MPTC), the carboxylic acid is extracted with a lipophilic quaternary ammonium cation from the aqueous bulk phase into the micellar pseudophase. In the micelle the reaction of the carboxylic acid with the label occurs[6].

In the other study[6] it was found that optimal derivatization rates at 70°C and pH 7.0 could be obtained with a micellar system that consisted of 50 mM Arkopal N-130 and 36 mM THxABr, and this composition was adopted throughout this study.

Derivatization in plasma

Effect of proteins. When plasma samples were incubated at 70°C in the micellar solutions, problems occurred with the derivatizations owing to the precipitation of the plasma proteins. The plasma proteins could in principle be removed prior to the derivatization, *e.g.*, by the addition of acids (*e.g.*, perchloric acid) or organic solvents (*e.g.*, acetonitrile or methanol)[9]. However, the addition of these deproteinizing reagents to the plasma sample strongly affects the micellar system and thereby the derivatization rates[6].

Another, more general, problem with deproteinizing procedures is that VPA, like many other drugs, is strongly protein bound (80–90%)[3] and could coprecipitate with the proteins. This makes the quantitative determination of VPA more difficult. We tried to circumvent the deproteinization step by simply diluting the plasma sample with buffer prior to the derivatization step in the micellar system. With a plasma content of 25% or less in the micellar solution only a slight or no protein clotting at 70°C was observed. Therefore, in further incubations a plasma content of 20% in the micellar solution was used.

Effect of BrMMC concentration. The derivatization of VPA in 20% plasma (method 1) using 1.5 m*M* BrMMC gave only a *ca.* 60% yield of the VPA derivative compared with derivatizations in buffer and acetone. In addition, it was found that in plasma, in contrast to buffer and acetone, the BrMMC was completely absent after the incubation. The obvious reason for this low recovery is that in plasma other compounds are present, such as free fatty acids (FFA)[10], which are derivatized with BrMMC. It was considered that with plasma the BrMMC concentration in the micellar solution had to be increased in order to ensure the presence of an excess of BrMMC during the introduction. However, it was recognized that with derivatization method 1 the reagent concentration could hardly be increased, owing to the limited solubility of the reagent in organic solvents and to the inhibiting effect of these solvents on the derivatization reaction in the micellar system[6]. Therefore, we evaluated other possibilities of adding the reagent to the micellar solution.

A major problem is caused by the low rate of dissolution of crystalline BrMMC in the micellar solution. However, if the BrMMC was precipitated in the presence of the surfactant and the ion-pair reagent from acetone (see Experimental), then the label readily dissolved after the addition of water to the incubation vial. An additional advantage of this new approach is that the BrMMC is stable in these prepared incubation vials for at least 1 month if stored at −20°C. Therefore, the incubation vials can easely be prepared in advance in large amounts and the actual sample preparation (method 2) is rapid and simple.

Fig. 1 shows the influence of the BrMMC concentration on the yield of the derivatization (method 2, *n* = 4) of 350 µ*M* VPA in plasma (100 µl) that was diluted with buffer (400 µl). The yield in plasma is expressed as a percentage of those obtained in pure aqueous micellar solutions and acetone. Fig. 1 shows that the yield of the VPA derivative reaches *ca.* 100%, which indicated that both the free and the protein-bound fractions of the VPA are determined. Apparently the bond between the VPA and the proteins is completely disrupted during the derivatization.

From Fig. 1 it can also be concluded that with a 20% plasma solution at least *ca.* 4 m*M* BrMMC is required for a 100% yield of the VPA derivative. In subsequent experiments we used a BrMMC concentration of *ca.* 5.3 m*M* in the incubation mix-
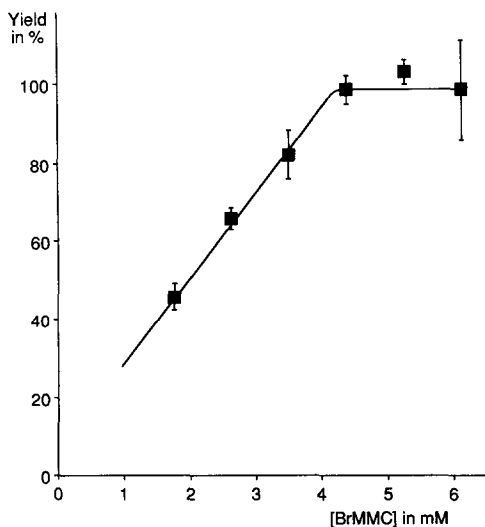
Fig. 1. Influence of BrMMC concentration on the yield of the derivatization of 350 μM valproic acid in plasma using incubation method 2.

ture to ensure the presence of an excess of the label during the incubation. At BrMMC concentrations higher than *ca.* 6 mM problems with the solubility of the label in the micellar system occurred, leading to a lower reproducibility of the derivatization.

Derivatization rate. Fig. 2 shows the kinetics of the derivatization of 350 μM VPA in plasma with 5.3 mM BrMMC using the conditions described in method 2. Fig. 2 illustrates that the derivatization of VPA is completed within *ca.* 35 min. In addition, it shows that the derivative is reasonably stable under the incubation conditions.

The VPA derivative is stable for at least 16 h in the acetonitrile-diluted micellar solution at room temperature. If stored at $-20°C$ the same sample is stable for at least 1 month.

Linearity. The linearity of the determination in plasma for VPA concentrations from 7 to 1042 μM ($n = 12$) was investigated. The calibration graphs passed through the origin and had a correlation of $r = 0.9476$ without the use of an internal standard and $r = 0.9997$ if the peak-area ratio relative to 10-undecenoic acid was used.

Reproducibility. The coefficient of variation (C.V.) of the determination of VPA in plasma was examined. At a spiked concentration of 50 μM VPA the C.V. of the peak area was 7.4% ($n = 6$). A similar reproducibility was observed with sodium valproate (C.V. $= 7.2\%$, $n = 5$) and with the internal standards nonanoic acid (C.V. $= 7.6\%$, $n = 6$) and 10-undecenoic acid (C.V. $= 7.5\%$, $n = 6$). The reproducibilities of the peak areas was not substantially improved at higher concentrations of the acids. In spiked plasma the C.V. of the peak-area ratio relative to 10-undecenoic acid as an internal standard was 2.5% ($n = 6$) at a concentration of 670 μM VPA and 3.2% ($n = 6$) with 38 μM VPA. At a concentration of 670 μM VPA similar reproducibilities of the peak-area ratios (C.V. $= 2.9\%$, $n = 6$) were found with nonanoic acid as an internal standard.
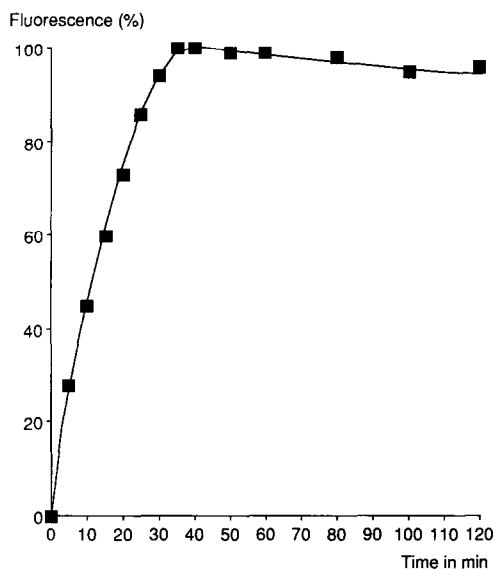
Fig. 2. Derivatization of 350 μM valproic acid in plasma with 5.3 mM BrMMC using incubation method 2.

From these results, it can be concluded that the use of an internal standard is advisable. However, the choice of the internal standard seems not to be critical.

Limit of detection. The limit of detection (LOD) of VPA in spiked plasma and buffer was investigated. In plasma the LOD was *ca.* 7 μM, owing to the presence of small interfering peaks in the chromatogram. For the monitoring of VPA at therapeutic levels (260–840 μM) this LOD value is sufficient. In buffer the LOD was *ca.* 25 nM. This shows that with this derivatization technique the carboxylic acids could in principle be detected sensitively without the need for tedious sample preparation procedures.

Patients' samples

The VPA concentrations in fifteen patients' samples were determined with the HPLC procedure using method 2. The chromatogram of such a derivatization is shown in Fig. 3a and that of a healthy subject in Fig. 3b. From these chromatograms it can be concluded that both VPA and undecenoic acid are well separated from the other plasma constituents.

The levels determined were compared with those obtained using a standard fluorescence polarization immunoassay (FPIA) in the clinical laboratory (Fig. 4). The correlation between the two methods is satisfactory ($r = 0.980, n = 15$). The relationship between the VPA concentration determined with the HPLC method, $[VPA]_h$, and that determined by FPIA, $[VPA]_f$, is $[VPA]_h = -7 \mu M + 1.01[VPA]_f$. At a VPA concentration of 590 μM the C.V. of the peak-area ratio in the patients' samples was 2.9% ($n = 5$). At the same concentration the reproducibility of the FPIA method was 2.5%.

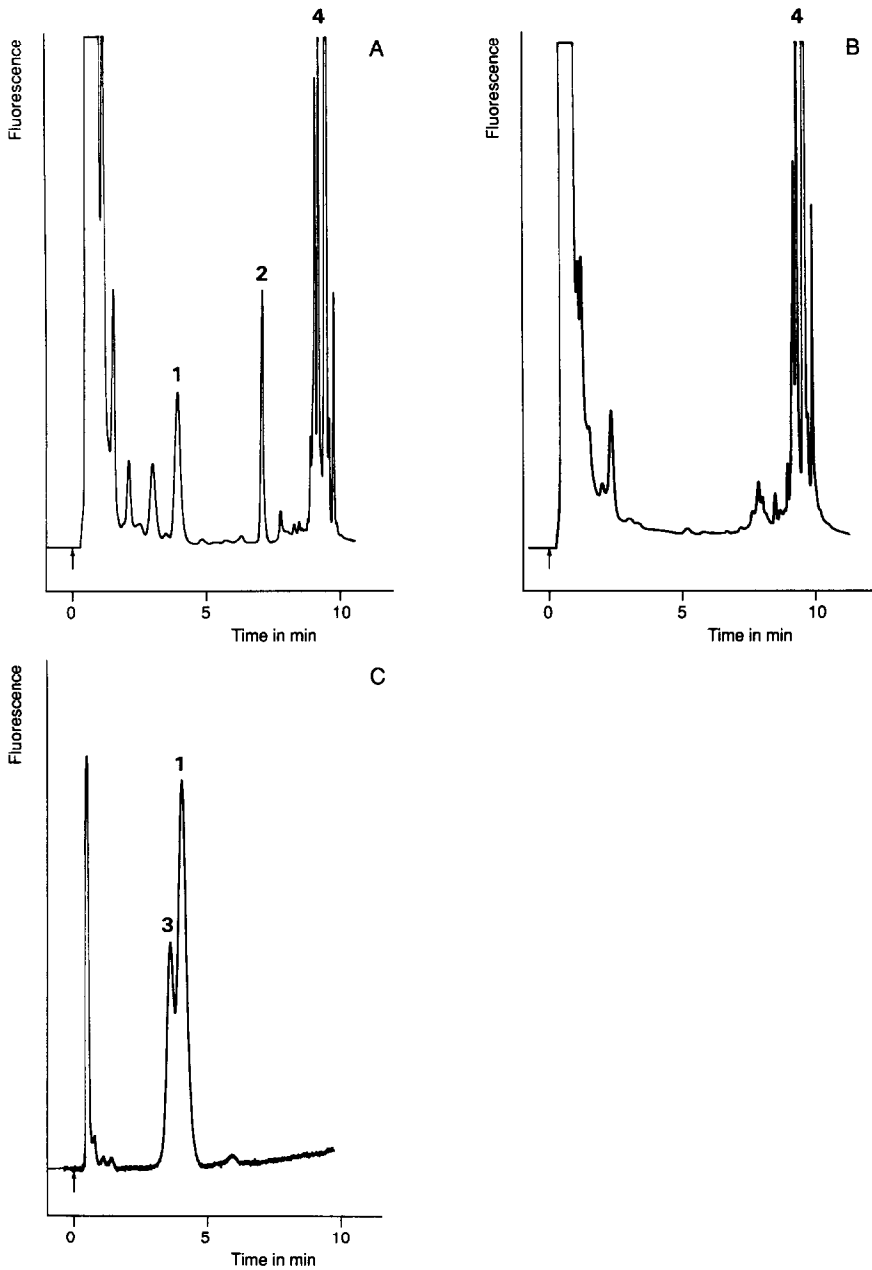The main advantage of HPLC over the FPIA method is the possibility of

Fig. 3. Chromatograms obtained after derivatization (method 2) of: (A) sample from patient under treatment with VPA with UA (250 μM) as internal standard; (B) plasma from a healthy subject; (C) synthetic (E)-2-en-VPA and VPA. Peaks: 1 = VPA; 2 = 10-undecenoic acid; 3 = (E)-2-en-VPA; 4 = plasma-free fatty acids.
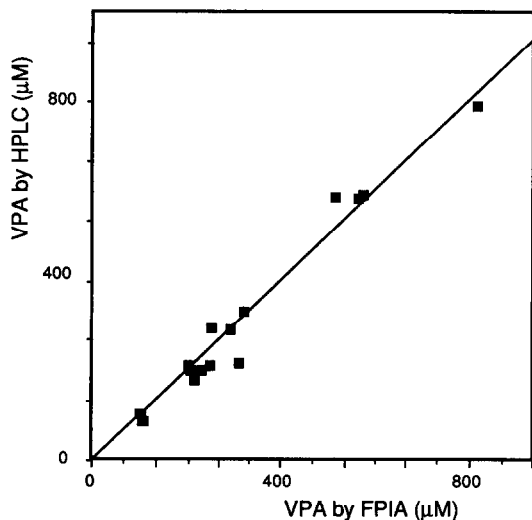
Fig. 4. Comparison of the FPIA and HPLC methods for the determination of VPA. VPA concentrations were determined (n = 15) in plasma samples from patients under VPA treatment. The solid line indicates the best least-squares fit (r = 0.980) between the results of the assays.

determining several bio-active compounds simultaneously. With VPA, especially its metabolites are of clinical importance. Normally only small amounts (< 8%) of metabolites of VPA are present in plasma [mainly (E)-2-propyl-2-pentenoic acid and 3-oxo-2-propylpentanoic acid][11,12]. However, elevated metabolite levels in plasma might be related to fatal liver injuries[12]. The deviations in the metabolite pattern could easely be monitored by the HPLC method. Although this field of research is outside the scope of this study, we performed some tentative experiments on the detection of one of the main metabolites of VPA in plasma, namely (E)-2-propyl-2-pentenoic acid [(E)-2-en-VPA][11,12]. Synthetic (E)-2-en-VPA was derivatized (method 2) and determined by HPLC (Fig. 3c). It can be seen that the (E)-2-en-VPA derivative and the VPA derivative eluted separately. Additional studies need to be performed, however, to investigate whether the HPLC method is suitable for monitoring the pathological VPA metabolism in patients' samples.

Some of the patients were also treated with other anti-epileptic drugs (e.g., phenobarbital, phenytoin and carbamazepin). We therefore investigated whether the presence of these compounds interfered with the determination of VPA. In contrast to carbamazepin, both phenobarbital and phenytoin gave a fluorescent derivative after reaction with BrMMC (method 2). This observation is in agreement with other studies[13,14] in which barbiturate-like compounds were derivatized with BrMMC in aprotic solvents. However, the derivatives of phenobarbital and phenytoin eluted near the void volume of the column and therefore did not interfere in the determination of VPA.

CONCLUSIONS

This study of the derivatization of VPA in plasma has shown that MPTC is a useful derivatization procedure for biologically active carboxylic acids. The main

advantage of MPTC over conventional derivatization procedures for carboxylic acids is that the extraction of the drug into a suitable organic solvent prior to the derivatization step can be omitted. This makes the determination of carboxylic acids faster and easier to perform.

Another interesting advantage of MPTC is that in principle it is suitable as an on-line derivatization procedure for carboxylic acids. However, with BrMMC as a label too long derivatization times are required for convenient interfacing with liquid chromatography. In plasma the precipitation of proteins at elevated temperatures would cause additional problems. We are now investigating MPTC systems that perform well at ambient temperatures, by means of which a automated on-line derivatization procedure for carboxylic acids is possible.

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