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Note

Automated analysis procedure for valproic acid in blood, serum and brain dialysate by high-performance liquid chromatography with bromomethylmethoxycoumarin as fluorescent label

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Valproic acid (2-propylpentanoic acid) is widely used as an anticonvulsant and has been applied in psychiatric conditions such as manic-depressive psychosis and tardive dyskinesia [1,2]. Monitoring of the blood levels of valproic acid may be useful to optimize clinical treatment; in the treatment of epilepsy this is done for plasma on a routine basis [3,4]. For the clinical effects to occur penetration into the central nervous system is required, and the free drug concentration in the brain in particular may be important for its effectiveness. Here we present a novel approach to measure the free drug concentration in the rat brain.

Several assays have been described for the analysis of valproic acid, including immunoassay and procedures using gas and liquid chromatography [5–9]. Direct detection of valproic acid after high-performance liquid chromatographic (HPLC) analysis is troublesome because of the lack of chromophoric or fluorescent groups. On the other hand, derivatization procedures generally require extraction and evaporation, which influence the reproducibility.

Recently we published a method for the automated HPLC analysis of fatty acids in plasma and rat brain homogenates after derivatization with bromomethylmethoxycoumarin (BrMMC) at room temperature [10]. The solid base and elevated temperature normally used in the reaction of acids with BrMMC can be avoided by using a suspension of potassium carbonate in the crown ether, 18-crown-6. Sample preparation for the analysis of valproic acid is reduced to addition of the biological matrix, e.g. serum, plasma or blood, to acetonitrile containing the internal standard, nonanoic acid ($C_{9\,0}$). The supernatant is added to a sample vial together with the suspension. The label is automatically added 15

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min to prior the analysis. The brain dialysate samples are analysed manually. In this way a smaller sample volume is required.

EXPERIMENTAL

Reagents and chemicals

All reagents were used as described before [10]. Nonanoic acid and valproic acid were obtained from Sigma (St. Louis, MO, U.S.A.). Animals were injected with a commercially available soluble mixture of sodium valproate (300 mg/ml; dose 25 mg per rat).

Apparatus

Autosampler. The PROMIS autosampler (Spark, Emmen, The Netherlands) was equipped with a digital dispenser system, a miniature 'finger pump'. A coaxial needle pair was inserted into the sample vial. Nitrogen pressure, applied through the outer needle, ensured that no air or vapour bubbles were formed during sample withdrawal. A commercially available two-way dispenser system was used, one line for the sample–suspension mixture and the other for the BrMMC solution. The ratio of label to suspension–sample was 1:1.

HPLC equipment. Two Kratos SF400 pumps and a Kratos SF450 gradient mixer were used for solvent delivery. For detection a Kratos SF980 fluorescence detector with a 5-μl cell was used (excitation wavelength 325 nm, cut-off filter 398 nm). Data were collected with an LDC CI-10 integrator (Interscience, Breda, The Netherlands). The separations were performed on a Chromspher ODS column (200 mm$\times$3 mm I.D., particle size 5 μm) (Chrompack, Middelburg, The Netherlands). Acetonitrile–2.5 *M* formic acid (75:25) was used for the separation. Elution was performed at a flow-rate of 0.4 ml/min at ambient temperature.

Dialysis. U-shaped cellulose dialysis fibres (200 μm O.D., 20 000 MW cut-off) were bilaterally implanted in the rat striatum one or two days before the experiments, as described in ref. 11. The rats (male Wistar, 200–250 g body weight) were obtained from the Centraal Proefdieren Lab. (Groningen, The Netherlands). The animals were perfused with artificial cerebral spinal fluid (CSF), containing 120 m*M* sodium chloride, 15 m*M* sodium hydrogen carbonate, 5 m*M* potassium chloride, 1.5 m*M* calcium chloride and 1 m*M* magnesium sulphate at a flow-rate of 1.4 μl/min. A pH of 7.4 was obtained by constant flushing with oxygen–carbon dioxide (95:5). Fractions were taken every 10 min for analysis. Valproate was injected through a cannula implanted into the heart. Through the same cannula 20-μl blood samples were taken at regular intervals to assay the blood levels of valproate.

Plasma, blood and dialysis samples

To 20 μl of plasma or blood, 1 ml of acetonitrile containing the internal standard nonanoic acid (1 μg/ml) was added. After centrifugation, 100 μl of the supernatant were added to a sample vial of the autosampler, together with 100 μl of suspension. The dialysis samples were lyophilized.

Derivatization procedure

To a sample vial of the autosampler, 100 μl of the supernatant of the serum or blood samples were added together with 100 μl of suspension. The suspension was prepared by adding 100 mg of potassium carbonate and 50 μl of water to 5 ml of a 20 m*M* solution of 18-crown-6 in acetonitrile. After 1 h of sonication, 5 ml of acetonitrile were added. The suspension was separated from the precipitated potassium carbonate; 15 min before injection the sample–suspension was automatically mixed with the label BrMMC (0.5 mg/ml), the injection loop serving as reaction coil.

The dialysis samples were analysed manually. To the lyophilized samples 40 μl of suspension with twice the amount of crown ether and 40 μl of BrMMC were added. The samples were thoroughly mixed and injected 15 min later.

Clinical analysis

On a routine basis valproic acid is determined by a polarization fluorescence immunoassay (PFIA, commercially obtained from Abbott Labs., Diagnostics Division, North Chicago, IL, U.S.A.).

RESULTS AND DISCUSSION

Derivatization and separation

A procedure based on the automated derivatization of fatty acids at room temperature [10] can be used for the detection of valproic acid. The separation of the methylmethoxycoumarin ethers of valproic acid and the internal standard is simple and straightforward under isocratic conditions (acetonitrile–2.5 *M* formic acid, 75:25) on a C_{18} column in 25 min (Fig. 1A). In an earlier stage of our investigations a substantial memory effect was noticed, which may be due to absorption of the unreacted fatty acids on the top of the column. With the subsequent injection a part of the absorbed fatty acids will be derivatized. Addition of formic acid, 2% per litre of eluent, clears this problem. The memory effect was less than 0.1% upon injection of 1 μg. The 0.5% of biological matrix per injection neither influences the separation nor causes deterioration of the column for at least 500 injections.

The double peaks of all acids are formed by an impurity in the BrMMC (details will be published). Structural analyses will be performed to elucidate the nature of the impurity, which has a very high fluorescence quantum yield that is not dependent on the composition of the mobile phase, i.e. the amount of water present, in contrast to BrMMC. Chemical characterization of the impurity may reveal a highly sensitive label for fatty acid.

Linearity and reproducibility

The calibration curve (Fig. 2) is linear from 1 to 500 μg/ml for all matrices tested with fluorescence as detection mode. In our system the upper limit of detection is not reached by the reaction with BrMMC but rather by the capacity of the integrator. Without this limitation concentrations up to at least 5 mg/ml valproic acid in serum can be measured. The standard deviation of the determination for serum samples containing 50 μg/ml was 1.3% ($n=6$). UV detection

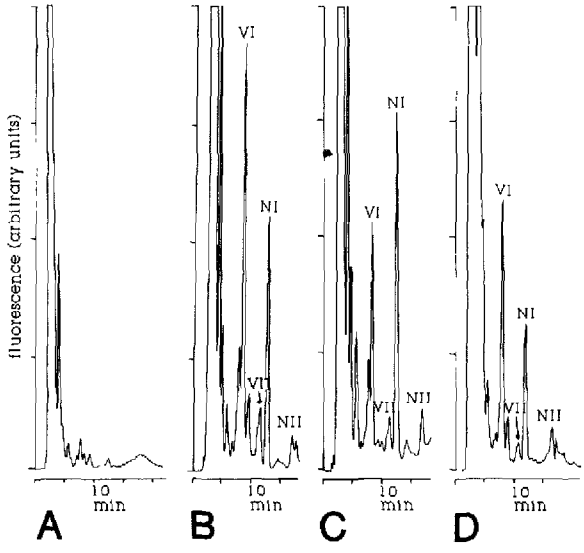

Fig. 1. Chromatograms of (A) blank human serum, (B) valproic acid in human serum, (C) rat blood and (D) rat brain dialysate, with the internal standard nonanoic acid. The first and second peaks are denoted as VI and VII for valproic acid and NI and NII for nonanoic acid, respectively. Mobile phase, acetonitrile–2.5 *M* formic acid (75:25); flow-rate, 0.4 ml/min; ambient temperature; fluorescence detection, excitation 325 nm, cut-off filter 398 nm.

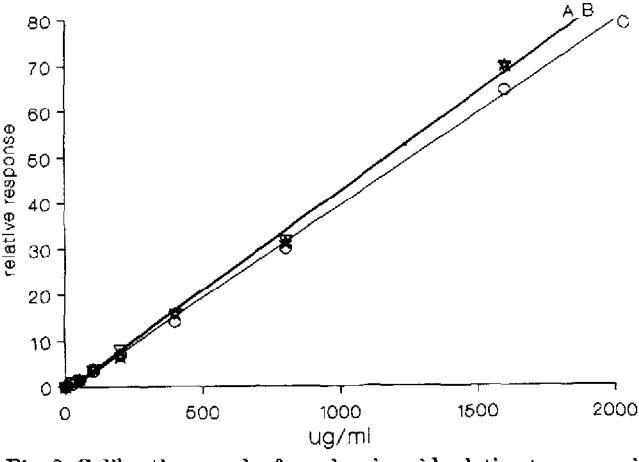

Fig. 2. Calibration graphs for valproic acid relative to nonanoic acid ($n=8$). (A) Acetonitrile; (B) human serum; (C) rat blood.

with this label is also possible. Although the detection limit will increase ten-fold, it is still in the therapeutic range.

Analyses of biological samples

Serum. The simple procedure for sample preparation, dilution of the serum with acetonitrile and centrifugation, is very suitable for clinical practice. The therapeutic range for valproic acid, 50–150 μg/ml of serum, lies within the linear

range of the calibration curve, taking the dilution into account. A typical chromatogram for serum containing valproic acid in the therapeutic range is shown in Fig. 1B.

The present approach also allows the analysis of the acid metabolites of valproic acid. The major metabolites of valproic acid found in serum of epileptic patients are 3-oxo-2-propylpentanoic acid and 2-propyl-2-pentanoic acid [12]. The metabolites are more polar, and so have lower relative retention times than the parent compound. Therefore no interference is to be expected with the valproic or internal standard peak.

The present method was compared with FPIA. A scatter diagram for the two methods is given in Fig. 3; the correlation coefficient is 0.98 and the angle between the correlation line with the x-axis is 44°. The comparison results in an S.E.M. of 0.6% ($n=24$).

Blood. Fig. 1C shows a chromatogram of the analysis of valproic acid in rat blood. To obtain information on the distribution of valproic acid between blood and the extra-cellular brain compartment, rats were injected into the heart with 25 mg of valproic acid. Fig. 4 shows the data for two rats for the first hour after drug administration.

Brain dialysate. In the same experiment described above, artificial CSF obtained from the striatum was analysed. The assay differed from that used for serum. Only shortly after mixing the dialysate with suspension a response with the label was seen. A reason can be that the derivatization reaction requires the initial production of the potassium salt of valproic acid. This salt is subsequently solvated by the crown ether [13], and the resulting complex will react with BrMMC to form the fluorophore. In the presence of sodium ions competition between the potassium and sodium ions may occur. Since the sodium salts of valproic acid are not solvated by the crown ether there will be no reaction.

There is a high coincidence between the time course in blood and in dialysate (an example of two experiments is given in Fig. 4). The dialysis efficiency for the flow-rate used (1.4 μl/min), determined in vitro, is 8.8%. Accordingly we calcu-

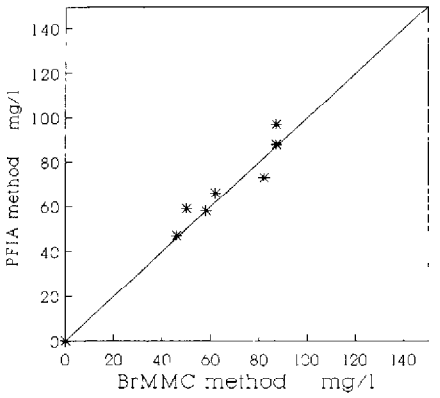

Fig 3. Comparison of results obtained for serum analysed for valproic acid by HPLC and by a polarization fluorescence immunoassay.

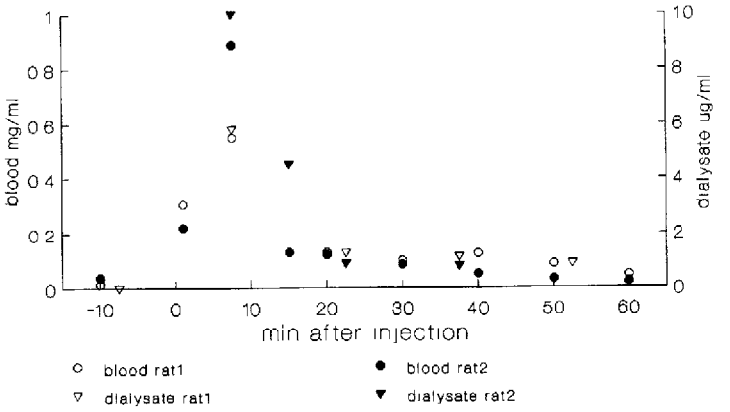

Fig. 4. Rat blood and dialysate concentrations of valproic acid for two rats after a single intravenous dose of 20 mg per rat intracardial.

lated a 3.5 times higher concentration in blood (average 3.51, S.E.M. 0.64, $n=10$) than in the extracellular compartment. (In this paper the emphasis is on the chemical methodology rather than on the pharmacokinetics, which will be described in more detail elsewhere.)

CONCLUSION

The automated precolumn procedure that we published [10] for the detection of fatty acids was used for the analysis of the anticonvulsant drug valproic acid in human serum, rat blood and rat brain dialysate. For serum and whole blood the procedure is simple and requires little pretreatment of the sample. For this reason the method is very suitable for routine drug monitoring. The blood can be taken by capillary puncture. Precolumn HPLC derivatization procedures are in general very suitable for clinical and biological investigations provided they can be automated. This method can be used for various acidic drugs, as long as they are soluble in media such as acetonitrile, acetone or dimethylformamide.

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