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PERFORMANCE EVALUATION OF A REVERSED-PHASE, HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF VALPROIC ACID INVOLVING A "SOLVENT DEMIXING" EXTRACTION PROCEDURE AND PRECOLUMN DERIVATISATION

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SUMMARY

As previously shown by others, the antiepileptic drug valproic acid can be assayed in biological fluids by reversed-phase, high-performance liquid chromatography after derivatisation with a bromomethyl aryl ketone through crown-ether catalysis. It is possible to extract the drug directly into acetonitrile, the solvent used for its derivatisation: when an excess of some salt such as NaCl is added to a mixture of plasma and acetonitrile, the organic solvent separates and valproic acid is extracted into it with a high recovery yield. This "solvent demixing" extraction method has shown excellent reproducibility, as well as promising versatility. Derivatisation, still in acetonitrile, using bromomethyl naphthyl ketone and 15-crown-5 allowed us to get rid of the current heating step without markedly increasing the delay of reaction. Chromatography was performed on a C-18 bonded stationary phase with acetonitrile—water as mobile phase, cyclohexanecarboxylic acid as internal standard and ultraviolet spectrophotometric detection. Statistical analysis of results shows 80% recovery of extraction, good linearity and an inter-extract variation coefficient of 4%, the last mainly ascribable to chromatographic measurements. Recovery is readily improved by increasing the amount of acetonitrile, which was equal to that of plasma in our experiments, since the high sensitivity of detection can tolerate the resulting decrease of valproic acid concentration in the extract.

INTRODUCTION

Valproic acid is a chemically simple drug (2-propyl-pentanoic acid, mol. wt. 144.2) used in the treatment of some types of epilepsy. Therapeutic levels in plasma or serum range from 260 to 840 μmol/l [1, 2] with a suggested threshold of efficiency at 350 μmol/l [3], of which 80—90% is protein-bound and

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which corresponds to daily dosages ranging from 15 to 30 mg/kg. Elimination half-life is estimated at between 13 and 21 h [2].

Opinions differ on the usefulness of blood assays intended for the therapeutic monitoring of this drug. Schobben et al. [1] doubt it, but a very well-planned study of Gram et al. [4] has recently shown that there is a good level–efficiency relationship between 110 and 350 μmol/l and that dosages required for obtaining a given level vary by more than double between individuals. These results, together with the observation of frequent subclinical toxicity to the liver which improves when doses are lowered [5], advocate the usefulness of assay.

Plasma levels of valproic acid are currently measured with gas chromatography [6—12]. Liquid chromatography is difficult owing to the low ultraviolet absorbance of this compound. Colorimetric detection using a pH indicator in the mobile phase has been proposed [13]. We turned to a general method of derivatisation of carboxylic acids by an aryl bromomethyl ketone with crown-ether catalysis [14]. This method using commercial reagents has already been proposed for the liquid chromatography of valproic acid [15] but extraction of the drug from plasma was not dealt with. A closely related method using amine instead of crown-ether catalysis has been recently published [16] which is said to perform as well as the gas chromatographic method [12] from which it stemmed.

We present a quantitative evaluation of a whole procedure applied to biological samples. Some original features are a rapid and reliable extraction method involving salting out of a water-miscible solvent (acetonitrile) and the suppression of heating the derivatisation mixture. We tried a smaller crown ether (15-crown-5) instead of 18-crown-6 currently used [14, 15, 17] merely because it was easier to obtain in this country.

MATERIALS AND METHODS

Reagents and solvents

Potassium chloride and carbonate Normapur, analytical grade, were from Rhone-Poulenc, (Paris, France). Acetonitrile for far UV was from Fisons (Loughborough, Great Britain) through Touzart et Matignon (Paris, France). Cyclohexanecarboxylic acid for synthesis was from Merck-Schuchardt (Darmstadt, G.F.R.). Valproic acid was kindly given by Labaz. Bromomethyl naphthyl ketone (α-bromo-2'-acetonaphthone, 99%) was from Aldrich (Milwaukee, WI, U.S.A.). 15-Crown-5 purum (1,4,7,10,13-pentaoxacyclopentadecane) was from Fluka (Buchs, Switzerland).

Working solutions

All solutions were made up in acetonitrile.

A solution of bromomethyl naphthyl ketone and a solution of 15-crown-5 both at twice the desired concentration were mixed in equal parts to make the so-called ketone—crown solution. In the same way a solution of valproic acid (VA) and a solution of cyclohexanecarboxylic acid (internal standard, IS), both at twice the desired concentration, were mixed to make the so-called VA—IS solution.

Chromatographic apparatus

An SP 8000 chromatograph from Spectra-Physics was used, equipped with a Valco loop injector (10 μl volume). The column was a Hibar RT 250-4 prepacked column from E. Merck (Darmstadt, G.F.R.) filled with LiChrosorb RP-18 bonded reversed phase, particle size 5 μm.

The detector was a Model 770 variable-wavelength, spectrophotometric detector from Schoeffel.

Solvent demixing extraction

In a 5-ml glass tube add successively 1 vol. (0.5 ml) of VA—IS solution (IS 0.40 mmol/l, VA either zero or at the indicated concentration), 0.1 vol. of 1 *N* HCl, and 1 vol. of water or plasma. Mix briefly by vortex (when plasma is used an homogeneous precipitate of proteins should be obtained). Add excess KCl (about 0.3 g) and mix vigorously (say ten brief vortex strokes at maximum speed) to saturate. Centrifuge (20°C, for at least 5 min at 1500 *g*); the extraction mixture separates into (from bottom upwards) excess KCl, a saturated aqueous layer, a compact disc of plasma proteins, and an acetonitrile layer of about two-thirds of the added volume.

Derivatisation

In a second tube add K_2CO_3 (about 20 mg) and 200 μl of acetonitrile supernatant. Mix by vortex to prepare potassium salts of the extracted acids (1 h). Add 200 μl of the ketone—crown solution, mix and leave to react at room temperature either for the time chosen (determination of the time course) or for 1 h to completion.

Chromatography

The reaction mixture was injected (10 μl) into an isocratic mobile phase of acetonitrile—water (83:17) at a flow-rate 1 ml/min at room temperature. Detection was made at 280 nm.

Experimental design and calculations

To determine the time course of derivatisation, salting out extractions from water were performed as described above with one VA—IS solution (VA 1.9 mmol/l, IS 0.40 mmol/l), derivatisation was carried out with three ketone—crown solutions at different concentrations indicated in the legend of Fig. 1, and the three derivatisation mixtures were chromatographed through time.

For the overall evaluation of the whole procedure, extractions were made both from water and from human serum with three VA—IS solutions (IS constant, VA as in Table I), each combination being replicated four times. Derivatisation was made with VA—IS solutions (subscript S), water extracts (subscript W) and plasma extracts (subscript P) and the $3 \times 3 \times 4 = 36$ samples were chromatographed following the order of numbers indicated in Table I to minimise any systematic factor of variation linked to time.

Peak heights (h) were measured on the chromatograms. The expected relationship

$$h = a\,C \qquad (1)$$

with concentrations C of VA may be written (decimal logarithms)

TABLE I

EXPERIMENTAL DESIGN FOR EVALUATION OF THE PROCEDURE

The 36 samples were chromatographed successively following the sequences given.

	IS; 0.40 mmol/l VA: 0.24 mmol/l	IS: 0.40 mmol/l VA: 0.48 mmol/l	IS: 0.40 mmol/l VA: 0.96 mmol/l
S*	1.18.19.36	2.17.20.35	3.16.21.34
W**	4.15.22.33	5.14.23.32	6.13.24.31
P***	7.12.25.30	8.11.26.29	9.10.27.28

*VA—IS solutions.
**Water extracts.
***Plasma extracts.

$$\log h = \log C + \log a \qquad (2)$$

Thus the linear regression log h vs. log C was expected to be a line of slope 1 if eqn. 1 was valid.

Under the assumption of a constant relative indetermination of measurements (a constant coefficient of variation of h), the logarithmic transformation warrants homogeneity of variance (homoscedasticity) needed for the regression to be valid.

Calculations were made as follows:

(1) Homoscedasticity of the 9 sets of 4 replications was first tested through Bartlett's test [18]. If not significant at the probability level $P = 0.1$,

(2) Three separate regressions (log h_S, log h_W, log h_p vs. log C) were then performed and their linearity was tested through one-sided F tests. If not significant at $P = 0.1$, analysis of the common regression log h vs. log C was started. Then

(3) Parallelism of the three lines was tested through a one-sided F test. If not significant at $P = 0.1$,

(4) Departure from 1 of the common regression coefficient was then tested through two-tailed t-test. If not significant at $P = 0.1$, the validity of eqn. 1 was admitted.

Further calculations included:

(5) Comparison of elevations [19, 20] of the lines corresponding to water or plasma extracts and to solutions in order to estimate the recovery of the extraction method, and

(6) An estimation of the overall coefficient of variation (C.V.) from the common residual variance s_C^2 about regression lines. This was made through the approximations

$$\text{C.V.} \approx \frac{dm}{m} = d \log_e m = 2.3026\ d \log_{10} m \approx 2.3026\ s_C$$

which assimilate ($\approx$) standard deviations with differentials and arithmetic with geometric means m, and which work well as long as C.V. is not too large, say lower than 10%.

RESULTS AND DISCUSSION

Time course of derivatisation

Fig. 1 shows the time course of derivatisation at room temperature. Of the three reagent concentrations tried, the highest one reached equilibrium after 1 h in a parallel manner for IS and VA. In fact, due to the parallelism of evolution, VA/IS peak ratios as used in an assay were stable after half an hour, which is not disproportionate to the 15-min reaction time proposed in the method of Durst et al. [14] and avoids heating at 90°C.

Values obtained with the two highest reagent concentrations did converge, not with the lowest one; this probably confirms that the reaction goes to completion if the bromoketone concentration is large enough [17]. Consequently, the proposed solvent demixing extraction, and particularly the probable hydration of the salted-out acetonitrile phase did not appear to affect the reaction seriously.

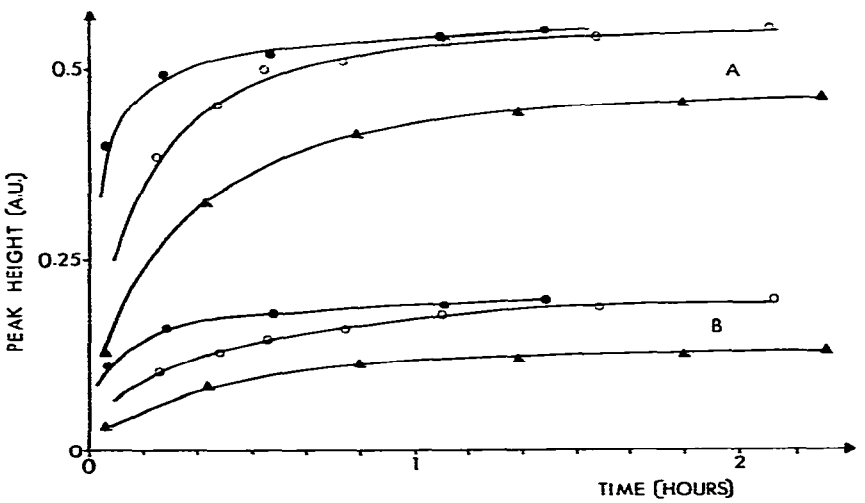

Fig. 1. Kinetics of derivatisation of valproic acid (VA) and of cyclohexanecarboxylic acid (internal standard, IS) with the following final concentrations of reagents in the reaction mixture: (A) valproic acid 1 mmol/l (corresponding to a plasma level of about 3 mmol/l or 300 mg/l); (B) internal standard 0.20 mmol/l; (▲) bromoketone 2.5 mmol/l, crown ether 0.188 mmol/l; (○) bromoketone 5 mmol/l, crown ether 0.375 mmol/l; (●) bromoketone 10 mmol/l, crown ether 0.75 mmol/l.

Evaluation of performance

Fig. 2 shows three typical chromatograms obtained with the VA—IS solution (a), a water extract (b) and a normal serum extract (c); no troublesome extraneous peak appears in the case of serum.

Fig. 3 illustrates logarithmic relationships obtained between VA concentrations and either peak heights h (A) or the ratio k of VA/IS peak heights (B), the variable used in assays with IS, which will be referred to as "peak ratio". Bartlett's test was significant in neither case ($0.10 < P < 0.25$ and $0.25 < P <$

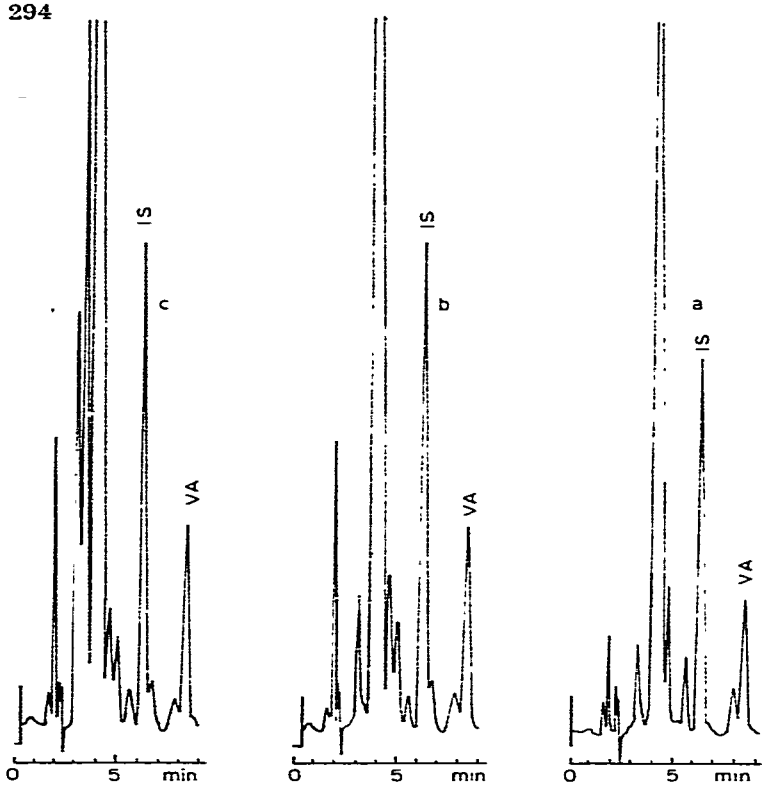

Fig. 2. Chromatograms of (from left to right): (c) an extract from normal serum; (b) an extract from water; (a) the VA—IS solution used for preceding extractions. Retention times of internal standard and valproic acid are 6.2 and 8.4 min, respectively.

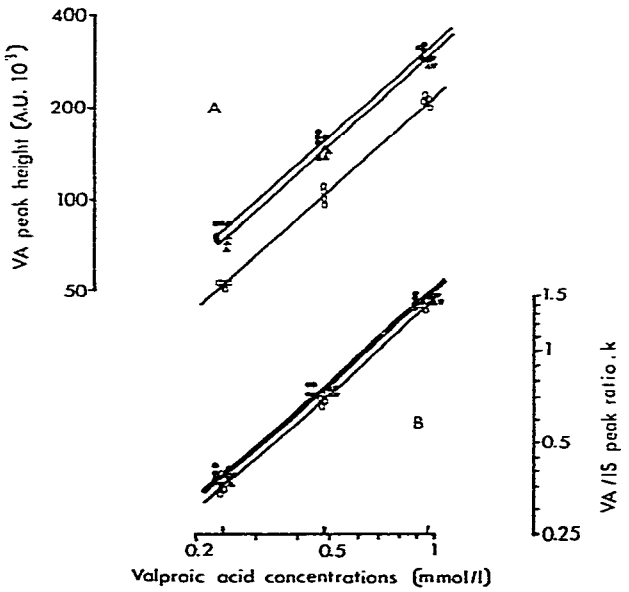

Fig. 3. Relationship between valproic acid concentrations (abscissa) and, in ordinate (both scales logarithmic): valproic acid peak height (A), and ratio of valproic acid over internal standard peak heights (B). (○), VA—IS solutions; (●), water extracts; (▲), plasma extracts. The sets of points corresponding to extracts have been slightly shifted from their true abscissa for the sake of clarity.

0.50 for h and k, respectively) which allows unweighted regressions to be performed.

Analysis of regressions log h vs. log C

Separate analysis of the three regressions (Fig. 3A) showed that none departed significantly from linearity ($F_{1/9} < 1$ in the three cases). Overall analysis showed that neither did the three lines depart significantly from parallelism ($F_{2/30} < 1$) nor did the common slope from one ($0.70 < P < 0.80$). The common residual variance was 0.00 04 98 12 (log unit)^2, corresponding to a 5.14% coefficient of variation, calculated as described in Methods.

Values obtained from water and plasma extracts were significantly ($P \ll 0.001$) higher than those from VA—IS solutions. This is due to the fact that the salted-out volume of acetonitrile was smaller than the added volume. The ratio of salted-out to added volume was separately measured on larger volumes and was found to be (mean ± standard deviation) $V_W/V_S = 0.613 \pm 0.02$ ($n = 9$), and $V_P/V_S = 0.593 \pm 0.012$ ($n = 9$). Comparison of elevations of the three parallel lines resulted in

$\log a_W - \log a_S = 0.1709$ $\quad a_W/a_S = 1.482$ (1.414—1.550)*

$\log a_P - \log a_S = 0.1415$ $\quad a_P/a_S = 1.385$ (1.322—1.448)*

*confidence interval 95%.

Thus the average yield of the described extraction method can be estimated to be: from water, (a_W/a_S) (V_W/V_S) = 91%; and from plasma, (a_P/a_S) (V_P/V_S) = 82%.

Analysis of regressions log k vs. log C

As above, for these regressions (Fig. 3B) statistical tests did not deny linearity ($F_{1/9} < 1$ in the three groups), parallelism ($F_{2/30} < 1$) and unit value of the common slope ($0.60 < P < 0.70$), so that the peak ratio k may be deemed proportional to VA concentrations in the range studied. The common residual variance about the lines was 0.00 02 82 94 (log unit)^2 corresponding to a 3.87% variation coefficient. Thus precision was not reduced, and was even better in fact, when using IS. This could mean that the elimination of variations of the injected volume (even with a loop injector) overcompensates for the double measurement of peak heights.

Unexpectedly, elevations were still significantly different ($P < 0.001$):

$\log a_W - \log a_S = 0.0376$ $\quad a_W/a_S = 1.0905$ (1.050—1.132)*

$\log a_P - \log a_S = 0.0312$ $\quad a_P/a_S = 1.0740$ (1.040—1.110)*

*confidence interval 95%.

This results shows that the extraction yield of IS was smaller than that of VA by nearly 10% and requires that the calibration of assays be made with standards extracted from a blank plasma.

Comments on the "solvent demixing" extraction procedure

The principle of this procedure was first applied by Bastos et al. [21] to the extraction of basic drugs from urine into ethanol through salting out with potassium carbonate, then extended to plasma extractions into isopropanol by Horning's team [22].

As already mentioned, variance was not significantly different among the nine sets of replications corresponding to solutions and to extracts. This means that the proposed extraction procedure did not impair precision significantly, i.e. that its reproducibility was good. This was to be expected, since the solvent is expelled from an homogeneous solvent—water mixture and the extraction yield is not exposed to such critical factors as duration of agitation, degree of dispersion, and interfacial "wetting", which can affect extractions into immiscible solvents. An incidentally useful consequence of initial homogeneity is that it does not matter that valproic acid be incorporated to the extraction solvent instead of the plasma; this feature greatly simplifies calibrations. The recovery was good for valproic acid. It has proved good too for other anionic anticonvulsants (phenobarbital, phenytoin, ethosuximide, carbamazepine) which we currently extract in the same way. A symmetrical basic extraction from plasma, using NaOH instead of HCl, was not so efficient for cationic drugs. It is probably a matter of adsorption on proteins, a difficulty currently met with with this kind of drug. Attempts to apply this method to other drugs and metabolites are in progress.

A further development of the "solvent demixing" principle is also under study: water demixing. The acetonitrile supernatant is transferred and a water-immiscible solvent, such as dichloromethane, is added to it in convenient amount (for example, 0.1 vol.). Then water, either acid or alkaline depending on the ionic species of the drug to be extracted, is added. Under mild motion, most of the acetonitrile dissolves in the water which results in a new two-phase system: water (and diluted acetonitrile) and dichloromethane. This method has shown promising efficiency and usefulness in preliminary experiments. Of course, solvent demixing extraction can be made more efficient if needed by increasing the volume of the water-miscible solvent, and more versatility by changing to other solvents.

Interferences

It is to be expected that all drugs with a carboxylic moiety are likely to react in the same way as valproic acid, and may interfere either with it or with the internal standard. Among the currently used antiepileptic drugs — ethosuximide, phenobarbital, phenytoin and carbamazepine — only phenobarbital appears to react with the derivatising reagent. Fig. 4 shows that the phenobarbital peak stands far enough apart from that of valproic acid not to impair measurement of the latter. This finding demonstrates, however, that reaction with a brominated ketone is not restricted to carboxylic acids.

Liquid vs. gas chromatography of valproic acid

We devised this method as an attempt at utmost technical uniformity in the routine determination of drug levels by reversed-phase liquid chromatography. However, gas chromatography (GC) still remains the current analytical stan-

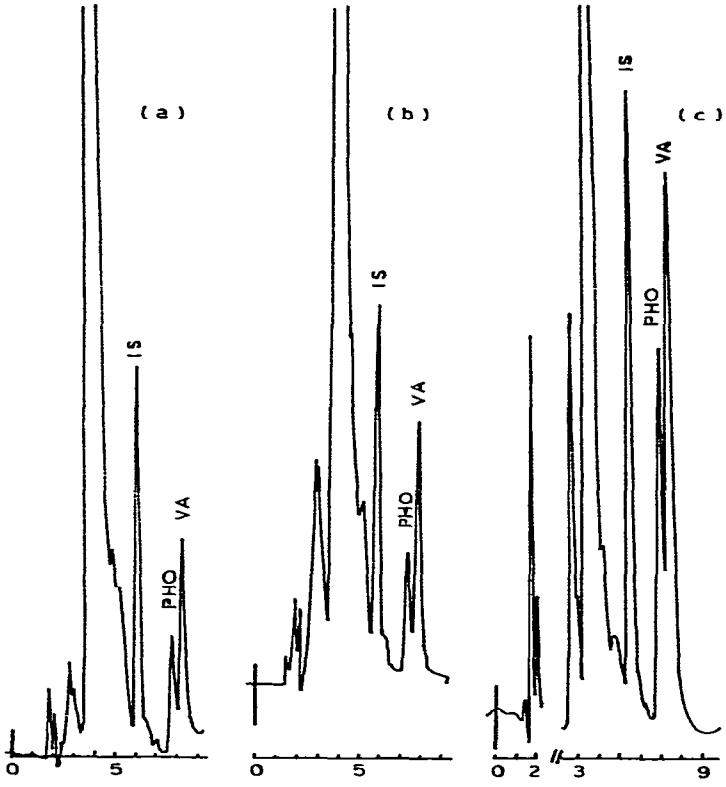

Fig. 4. Chromatograms of extracts from three plasma samples of patients receiving both valproic acid (VA) and phenobarbital (PHO). Levels were as follows (μmol/l), PHO being measured by another method: (a) VA = 330, PHO = 75; (b) VA = 460, PHO = 77.5; (c) VA = 590, PHO = 190.

dard. Gupta et al. [12] reviewed and solved the main difficulties met with by many authors in devising GC assays of valproic acid, the most troublesome being probably the instability of separation parameters due to the stationary phase being contaminated by extraneous organic solutes. This, together with the risk of evaporation losses of VA, led Gupta et al. to devise a method including both a purification step and derivatisation before concentrating the extract. The high absorbance of the aromatic derivative prompted them to apply their derivatisation procedure to liquid chromatography with "similar results" [16].

On technical grounds, the 1-h long sample preparation of Gupta et al. compares with ours, as does their 250-μl sample volume with our 500 μl. Perhaps our method is more liable to sample volume reduction than theirs, since we injected only 10 μl of our 400-μl derivatisation mixture, which allows us to use ten-times smaller volumes if necessary. Gupta et al. [12] did not state how much of their 50-μl final methanolic solution they injected. Finally, neither retention times nor peak shape showed any pejorative trend throughout about a thousand chromatograms. The column needs no washing after use, owing to the absence of any buffer in the mobile phase; nor does any clogging appear

from injecting acetonitrile extracts, owing to its high acetonitrile content. In fact, our columns usually die from channelization, which we are tempted to ascribe to mechanical strains exerted by our harshly regulated pumping device. As for the quality of routine determinations, accuracy can hardly be stated otherwise than through external quality control [23]. Our method shows an acceptable precision, though our 4% within-batch coefficient of variation is larger than the mere 1—2% of Gupta et al. [12], the latter estimation coming down, in fact, to the usual precision of the required initial double volumetric measurement of sample and internal standard solution.

CONCLUSION

Direct extraction into the solvent used for the derivatisation, suppression of heating and the possibility to perform calibrations with solutions made in the extracting solvent instead of using spiked plasmas, make the liquid chromatography of valproic acid quick and easy. Analysis of our results has shown that this simplification was not obtained at the expense of precision, and even that the proposed solvent demixing extraction combines simplicity and a high reproducibility. One point remains to be checked: how large is the variability of peak ratio k between different plasma samples? Owing to the incomplete (70—80%) extraction of valproic acid and of internal standard, it is not excluded, though improbable, that inter-plasma peak ratio variability may be larger than the mere intra-plasma component which was evaluated in this work. Should it so happen, extraction yield could be increased by increasing the amount of acetonitrile beyond the volume-for-volume ratio arbitrarily adopted here. This would dilute the extract and decrease the sensitivity, but the high ultraviolet absorbance of the naphthyl ketone allows an appreciable margin.

We are currently using this method for the therapeutic monitoring of valproic acid. Besides the usual reasons for such a monitoring, the suspicion that valproic acid could have a concentration-related metabolic toxicity seems to justify extensive studies.

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