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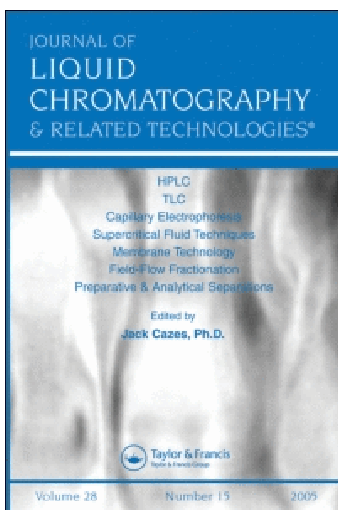

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Simultaneous Determination of Six Anticonvulsants in Serum by High Performance Liquid Chromatography

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SIMULTANEOUS DETERMINATION OF SIX ANTICONVULSANTS IN SERUM BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The authors describe a simultaneous determination method of six anticonvulsants (ethosuximide, primidone, phenobarbital, phenytoin, carbamazepine, valproic acid) using 500 µl of serum.

After the addition of the internal standard (5-(p-methylphenyl)-5 phenylhydantoin), the anticonvulsants are extracted in an acid medium with ethyl acetate. They are separated by reverse phase chromatography on a µ Bondapack C18 column, eluted with a water/methanol mixture (36/64 V/V) at a flow rate of 0.7 ml/min.

The column effluent is first analyzed by monitoring the ultraviolet absorption at 197 nm and then at 425 nm after the addition of a color indicator: bromocresol purple. The analysis lasts 12 minutes at ambient temperature. The sensitivity obtained with the serum for the range of products investigated is of the order of 0.5 to 2 mg/l, the extraction

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recoveries varying from 75 to 100% depending on the drug. Reproducibility is good (cv < 9%).

INTRODUCTION

The monitoring of anticonvulsant therapeutics has become a useful tool for the clinician and is usually carried out by gas chromatography (GC) (1,2) or by immunoassay (3). High performance liquid chromatography has special advantages for the analysis of these anticonvulsants when used during the course of a single treatment: previous transformation of the molecules is not necessary and simultaneous analysis is possible on the same column. Various methods have been proposed (4 to 8) but these restrict the determination of products detectable by spectrophotometry in ultraviolet to 195 nm (4,5,6) and 254 nm (7), and by fluorimetry (8). Having shown the possibility of separation on the same column and detection of chemical substances as different as phenobarbital and valproic acid by a colorimetric reaction at 425 nm (9), we propose to associate the ultraviolet and visible spectrophotometric detections. Hence it is possible to determine simultaneously from 500 μl of serum: ethosuximide, primidone, phenobarbital, phenytoin, carbamazepine and valproic acid. Good separation is obtained by reverse phase chromatography using a non-buffered mobile phase compatible with the colorimetric reaction. This is based on the acidic property of certain of these molecules with regard to a color indicator.

This method is rapid. It enables the accurate determination of quantities as low as 0.5 to 2 mg/l, and may be applied to the routine analysis of anticonvulsant drugs.

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MATERIALS AND METHOD

Apparatus

Biological samples and standard solutions were assayed using two Waters Associates 6000 A pumps equipped with a Waters Associates U6K injector and two variable wavelength spectrophotometers (Vari-chrom Varian and Dupont Instruments). The μ Bondapack C18 (4 mm x 30 cm) reverse phase column was eluted with 64% methanol/36% water (V/V) at a flow rate of 0.7 ml/min. The mixing chamber was a T Swagelock 1/16 inch filled with glass particles (40 to 70 μ). The response was recorded on a two-track Cole Parmer recorder (speed 30 cm/h).

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Figure 1 shows the schematic diagram of the apparatus. The mobile phase is introduced into the injector and column by the first pump. It then passes through the spectrophotometer where a reading is taken at 197 nm. It is immediately sent into the mixing chamber, where the bromocresol purple indicator solution is introduced. Absorbance is then measured at 425 nm.

Reagents and standards

Methanol RP for ultraviolet spectrophotometry (Prolabo, France).

Ethyl acetate RP (Prolabo, France).

Mobile phase. This is a degassed solution of 64 ml of methanol in 36 ml of bi-distilled water.

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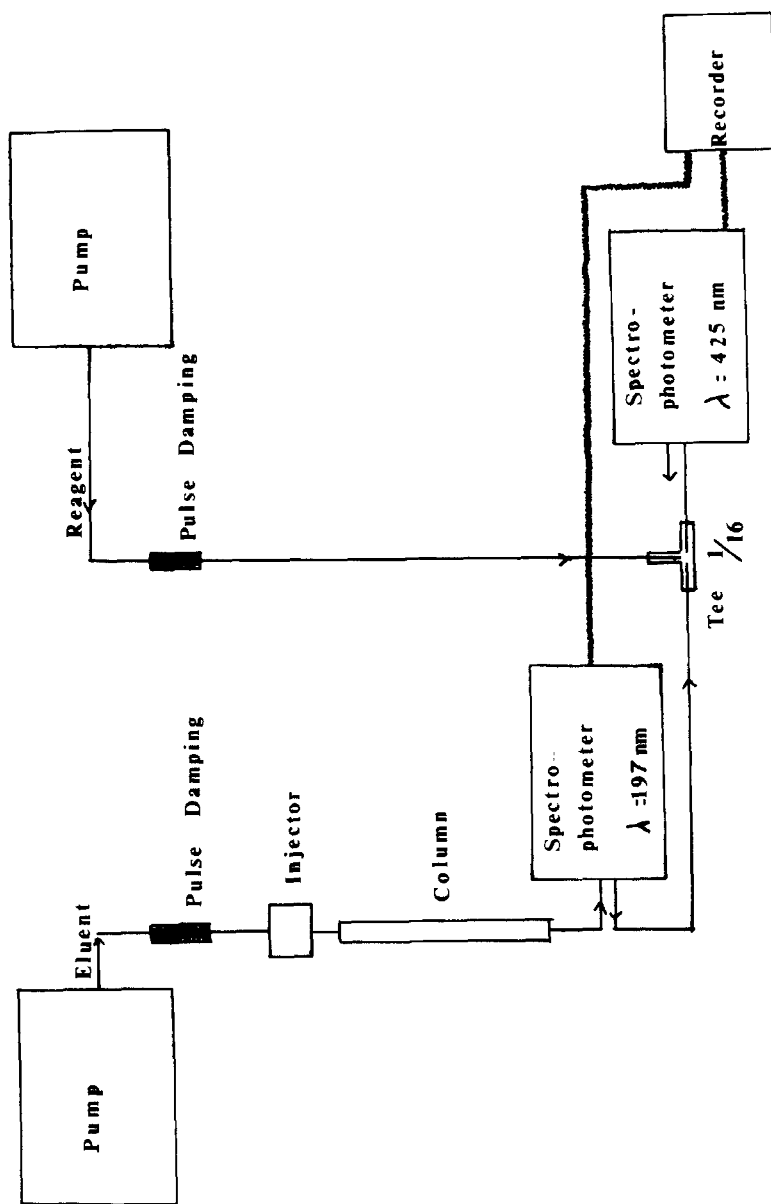

FIGURE 1 Schematic diagram of the system for the separation and detection of anticonvulsants

The indicator solution is prepared by dissolution of 40 mg of bromocresol purple in one liter of the mobile phase. The pH is adjusted to 7.5 by the addition of 1.5 ml of 0.1 N sodium hydroxide.

All other substances were of analytical grade.

The ethosuximide is supplied by Parke-Davis and the primidone by ICI Pharma, France.

We purchased the following:

. phenobarbital from Spécia,
. diphenylhydantoin from Laboratoire Carion, France,
. carbamazepine from Ciba-Geigy,
. sodium valproate from Berthier Derol, France,
. 5-(p-methyl-phenyl)-5 phenylhydantoin from Aldrich Chemical Company.

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Standard solutions

The standard solution of valproic acid (sodium salt) is an aqueous solution at 0.23 g/l corresponding to 0.20 g/l of valproic acid.

The internal standard solution is a methanol solution at 0.1 g/l.

The standard solutions of ethosuximide, primidone, phenobarbital, phenytoin and carbamazepine are prepared from methanol solutions at 2 g/l of each of the constituents.

Five standard solutions (I, II, III, IV and V) containing increasing concentrations of anticonvulsants are prepared as shown in Table 1. Variable volumes of each of the 2 g/l solutions are introduced into a 20 ml graduated flask. The final volume of 20 ml is obtained by adding methanol.

Method

500 µl of serum are introduced into a 5 ml glass-stoppered centrifuge tube. 100 µl of the internal standard solution are then added, together with 200 µl of N sulfuric acid and 2 ml of ethyl acetate.

The mixture is then shaken for a minute and centrifuged. The organic phase is collected, washed with 500 µl of 10^{-2} N sulfuric acid, dried with anhydrous sodium sulfate, and evaporated under nitrogen. The residue is dissolved in 50 µl of the mobile phase. 5 to 10 µl are introduced on the column. Table 2 gives the operating conditions for chromatography.

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TABLE 1

Preparation of standard solutions

	2 g/l solutions of:					methanol to
	ethosuximide	primidone	phenobarbital	phenytoin	carbamazepine	
solution I	1 ml	0.05 ml	0.25 ml	0.25 ml	0.05 ml	20 ml
solution II	2 ml	0.25 ml	0.50 ml	0.50 ml	0.25 ml	20 ml
solution III	3 ml	0.50 ml	1.00 ml	0.75 ml	0.40 ml	20 ml
solution IV	4 ml	1.00 ml	2.00 ml	1.00 ml	0.50 ml	20 ml
solution V	6 ml	1.50 ml	2.50 ml	1.50 ml	1.00 ml	20 ml

TABLE 2

Chromatographic conditions

column	µ Bondapack C18 4 mm x 30 cm
mobile phase	methanol/water (64/36 V/V)
flow rate	0.7 ml/min
indicator reagent	bromocresol purple 40 mg/l in the mobile phase
flow rate	1.8 ml/min
temperature	ambient
detector wavelength	. 197 nm . 425 nm

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Calibration is carried out in the same way: 100 µl of solutions I, II, III, IV and V (Table 1) are placed together with 100 µl of the internal standard in different glass-stoppered centrifuge tubes. After evaporation, variable quantities of the aqueous solution of sodium valproate are added, together with 500 µl of the serum.

Table 3 summarizes these various operations and indicates the final anticonvulsant serum concentrations for each tube.

RESULTS

Figure 2A shows the chromatogram of a serum loaded with the products under investigation and used for the calculation of retention times. Under the conditions mentioned above, ethosuximide, primidone, phenobarbital, valproic acid, phenytoin and carbamazepine have respectively the following retention times: 4.9, 5.3, 6.0, 7.2, 8 and 9.4 minutes.

TABLE 3

Preparation of the calibration range

drug	solution 100 µl				
	I	II	III	IV	V
	internal standard 100 µl				
	serum 500 µ l				
	sodium valproate				
	50 µl	100 µl	200 µl	300 µl	400 µl
	concentrations in serum (mg/l)				
ethosuximide	20	40	60	80	120
primidone	1	5	10	20	30
phenobarbital	5	10	20	40	50
phenytoin	5	10	15	20	30
carbamazepine	1	5	8	10	20
valproic acid	20	40	80	120	160

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As can be seen in Figure 2B, the normal serum constituents have in no way influenced the chromatogram.

Figure 3 shows a serum of a patient which has been extracted and chromatographed as described above.

We have studied the analytical recovery of drugs from serum by adding known quantities of ethosuximide, primidone, phenobarbital, phenytoin, carbamazepine and valproic acid to a pool of serum known to be free of drugs. The serums are extracted and the internal standard added after extraction.

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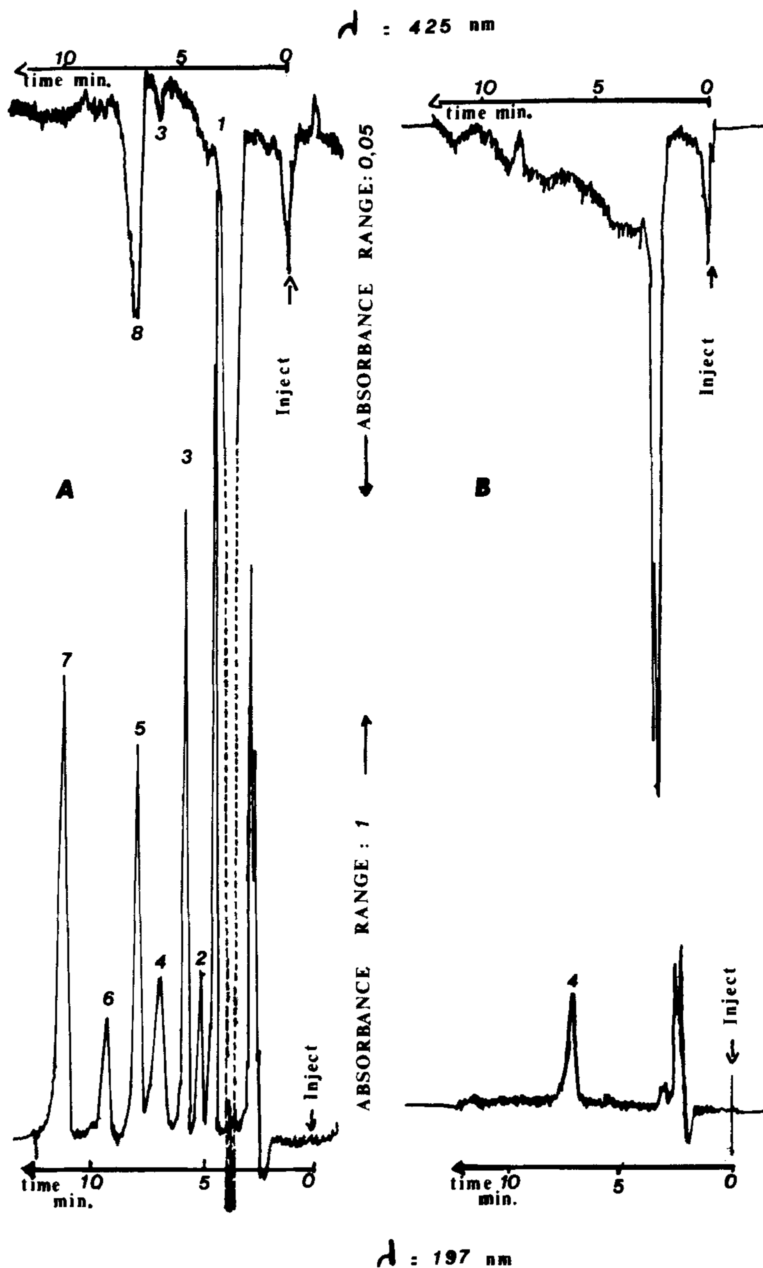

FIGURE 2 A: Chromatogram of an extract of a mixture of serum and drugs. Volume injected 5 µl. (1) ethosuximide 80 mg/l; (2) primidone 10 mg/l; (3) phenobarbital 20 mg/l; (4) non-identified; (5) phenytoin 15 mg/l; (6) carbamazepine 8 mg/l; (7) internal standard; (8) valproic acid 60 mg/l.
B: Chromatogram of drug-free serum

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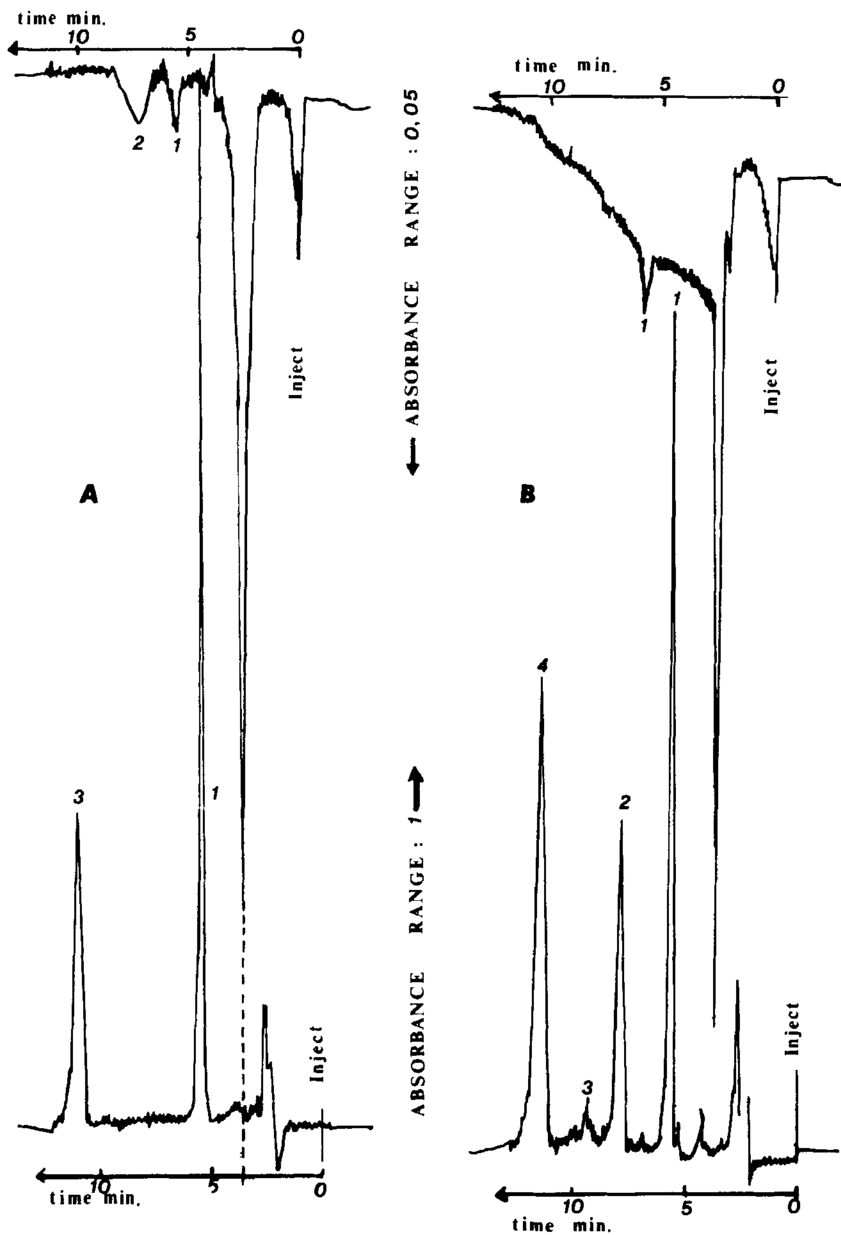

FIGURE 3 A: Chromatogram of a serum extract. Concentrations are calculated as follows: (1) phenobarbital 52.5 mg/l; (2) valproic acid 31 mg/l; (3) internal standard. B: Chromatogram of a serum extract containing: (1) phenobarbital 27.2 mg/l; (2) phenytoin 13 mg/l; (3) carbamazepine 2 mg/l; (4) internal standard

Results are expressed as percentages of the peak heights obtained by direct injection of pure standards. The results obtained for the different concentrations of each product with ether and with ethyl acetate are given in Table 4.

The investigation of linearity (Figures 4, 5 and 6) is carried out for therapeutic and toxic concentrations of the

TABLE 4

Extraction recoveries in ether and in ethyl acetate

drug	concentration (mg/l)	ether recovery (in %)	ethyl acetate recovery (in %)
ethosuximide	20	44	79
	60	47	81
	120	42	84
primidone	1	42	79
	10	37	74
phenobarbital	5	102	102
	20	100	99
	50	91	84
phenytoin	5	100	97
	15	98	98
	30	94	94
carbamazepine	1	54	87
	8	62	85
	20	55	80
valproic acid	40	96	95
	80	98	95

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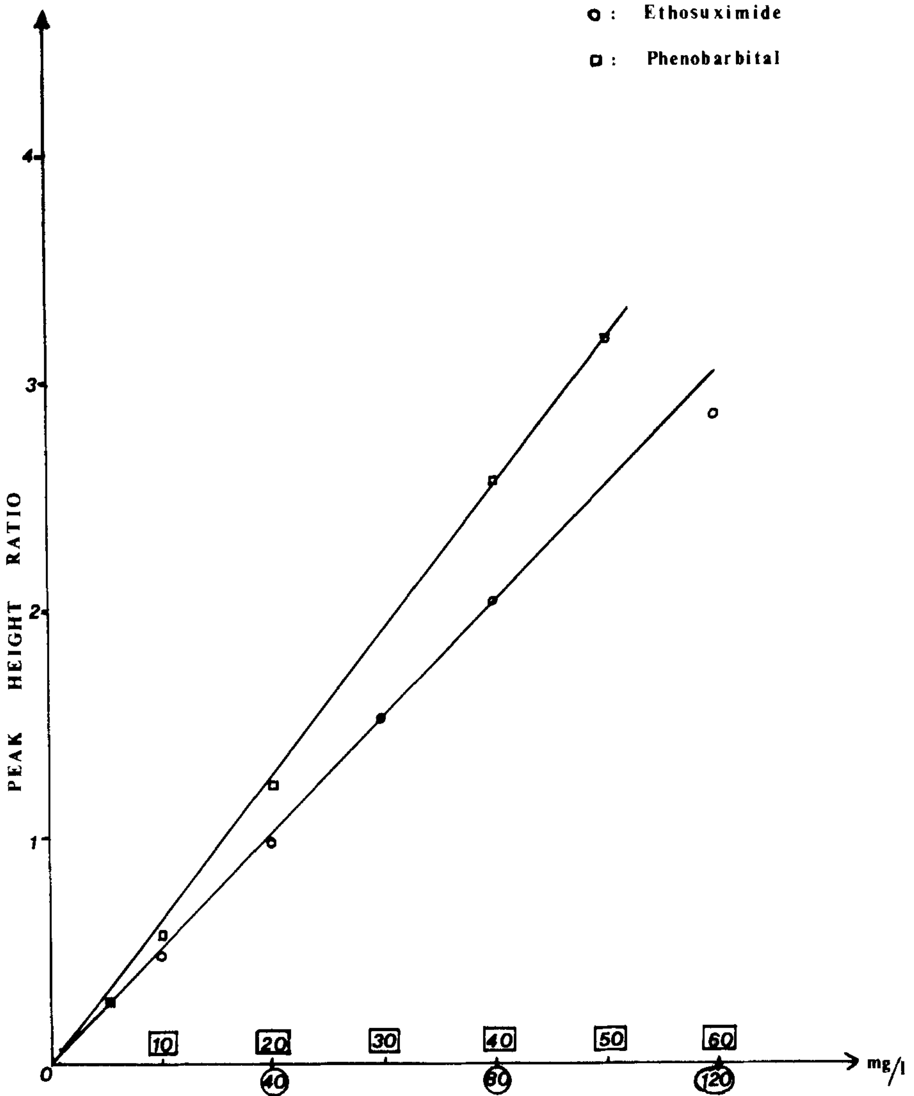

FIGURE 4 Peak height ratios (drug/internal standard) for ethosuximide and phenobarbital plotted versus concentration of each drug

various molecules examined (10). The results obtained show good linearity.

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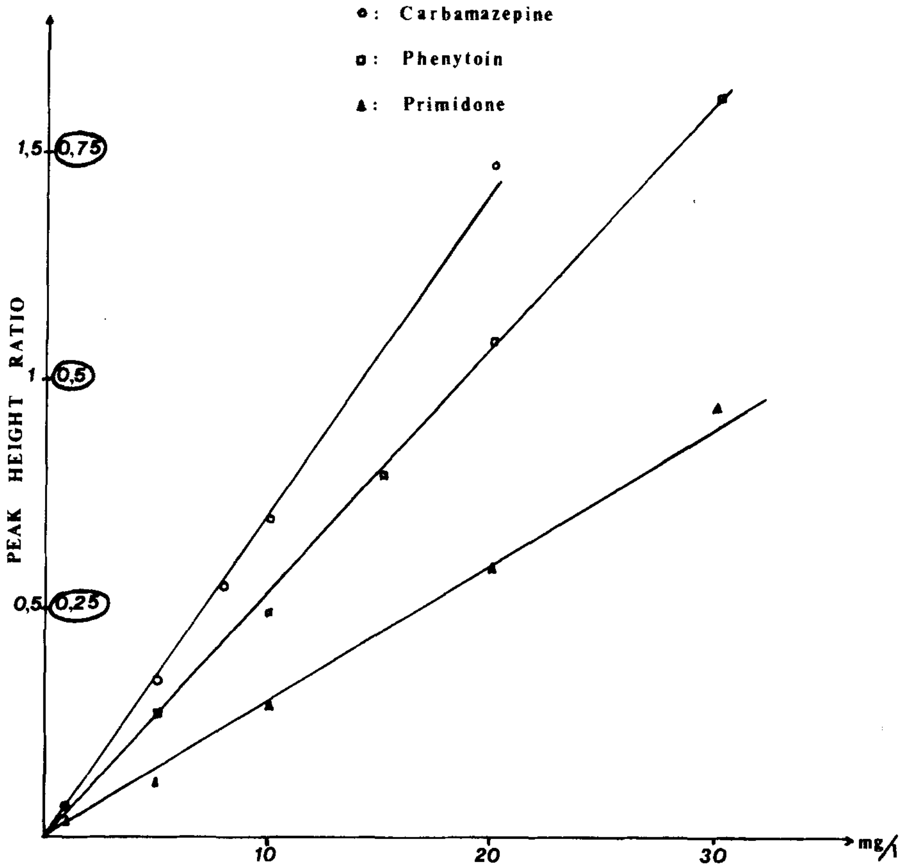

FIGURE 5 Peak height ratios (drug/internal standard) for primidone, phenytoin and carbamazepine plotted versus concentration of each drug

Table 5 gives the results obtained after ten extractions of a serum loaded with the products investigated. Good reproducibility using this method is shown.

The sensitivity of the method is limited by serum volume, extraction efficiency and background. With 500 µl of serum, the compounds can be detected in concentrations of 2 mg/l for

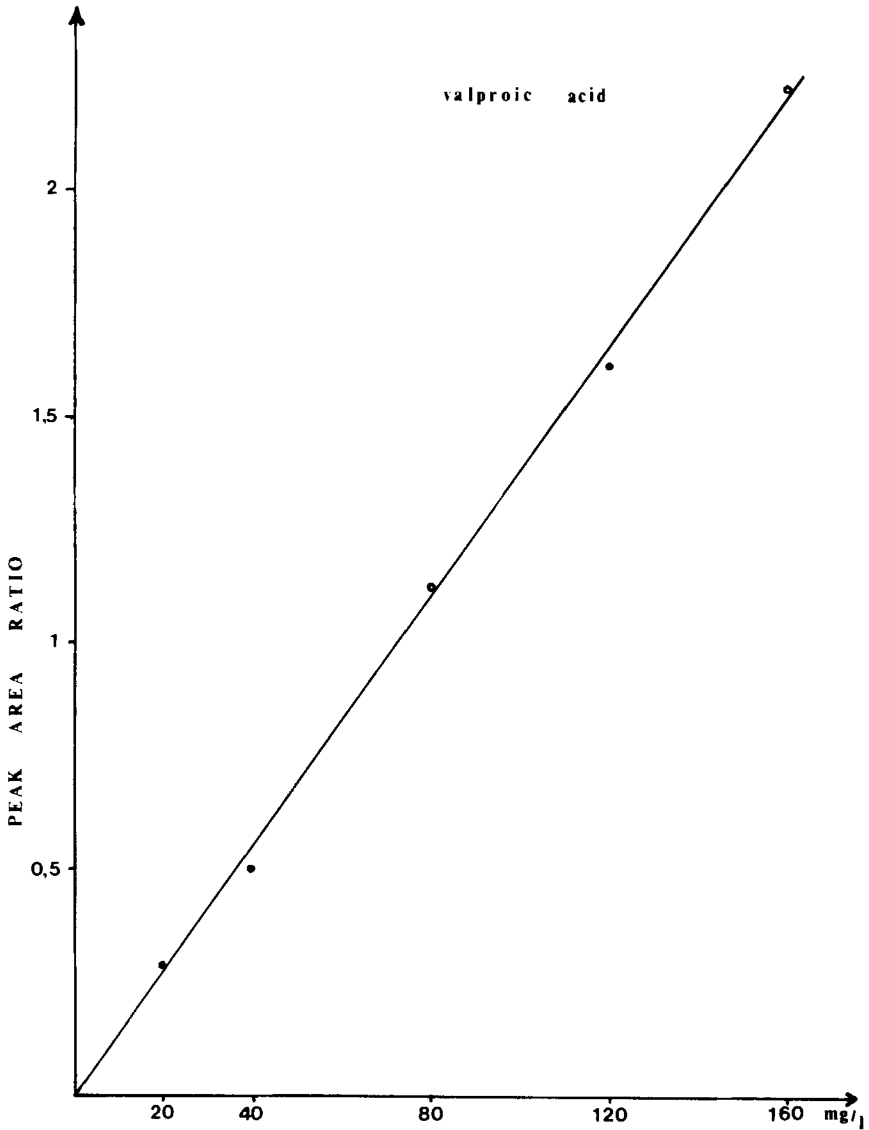

FIGURE 6 Peak area ratios (drug/internal standard) for valproic acid plotted versus concentration

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ethosuximide and valproic acid, and 0.5 mg/l for primidone, phenobarbital, phenytoin and carbamazepine.

TABLE 5

Reproducibility of the method

n	drug	CV %
10	ethosuximide	7.5
10	primidone	8.4
10	phenobarbital	3.8
10	phenytoin	3.0
10	carbamazepine	8.7
10	valproic acid	3.5

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DISCUSSION

Among various methods of HPLC recently proposed (4 to 8), we have not used the method of direct injection of deproteinized serum proposed by Kabra et al (4). We have chosen, rather, conventional extraction by an organic solvent (7). As Goedhart et al (11) show, in an acid medium, the coefficients of partition of the products investigated are better in ethyl acetate than in ether. This is in agreement with the results which we have obtained (Table 4) and which show extraction recoveries in ethyl acetate greater than 80%. Washing of the organic phase with an acid solution enables elimination of a certain number of absorbing impurities at 197 nm. Some serums present an impurity between phenobarbital and phenytoin (Figure 2) but this has no influence on the results. The evaporation of ethyl acetate should be slow in order to avoid possible entrainment of the valproic acid.

We have chosen reverse phase chromatography because this is easy to use and enables faster analyses than with

adsorption chromatography (7). We have investigated various binary mixtures (water/acetonitrile, water/methanol). The water/acetonitrile mixture requires the use of a buffer to obtain good separation of phenytoin and carbamazepine. In the present case, this is not compatible with the colorimetric detection method used. On the other hand, the water/methanol 36/64 V/V mixture at a flow rate of 0.7 ml/min gives good results, and enables separation at ambient temperature.

Except for valproic acid, all the molecules absorb ultraviolet, but the ethosuximide and primidone spectra show a sharp peak at 197 nm, which implies accurate retention of this wavelength. The colorimetric reaction adopted for the determination of valproic acid was chosen on the basis of two characteristics: the pK_a of bromocresol purple and its molecular extinction coefficient at 425 nm ($\varepsilon \approx 10^5$) (9).

The reagent flow rate and the dead volume of the mixing chamber exert an important influence on the broadening of the peaks. To reduce this, various tests have shown the necessity of maintaining the ratio:

$$\frac{\text{reagent flow rate}}{\text{mobile phase flow rate}} < 3$$

and of filling the mixing chamber with glass particles.

We have studied the risk of effects on determinations by several drugs extractible by ethyl acetate in an acid medium. Table 6 shows the retention times of separated products.

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TABLE 6

Retention times of various drugs extractible in an acid medium by ethyl acetate

compound	retention time (min)
salicylic acid	2.4
barbital	4.2
ethosuximide	4.9
primidone	5.3
phenobarbital	6.0
valproic acid	7.2
phenytoin	8.0
butobarbital	8.1
butalbital	8.1
amobarbital	9.8
pentobarbital	9.8
carbamazepine	9.4
secobarbital	10.0
5-(p-methyl-phenyl-5-phenylhydantoin	11.2

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The effect of butobarbital and butalbital on phenytoin can be seen and also that of amobarbital and pentobarbital on carbamazepine. This is a minor problem when monitoring treatment where each element is known.

Previously in our laboratory, we routinely determined phenobarbital, phenytoin and valproic acid by gas chromatography. This has enabled us to compare the two methods with these three compounds. Figure 7 gives the results for phenobarbital in 28 serums. The correlation coefficient is 0.98, slope 0.89, y-intercept 2.4 mg/l.

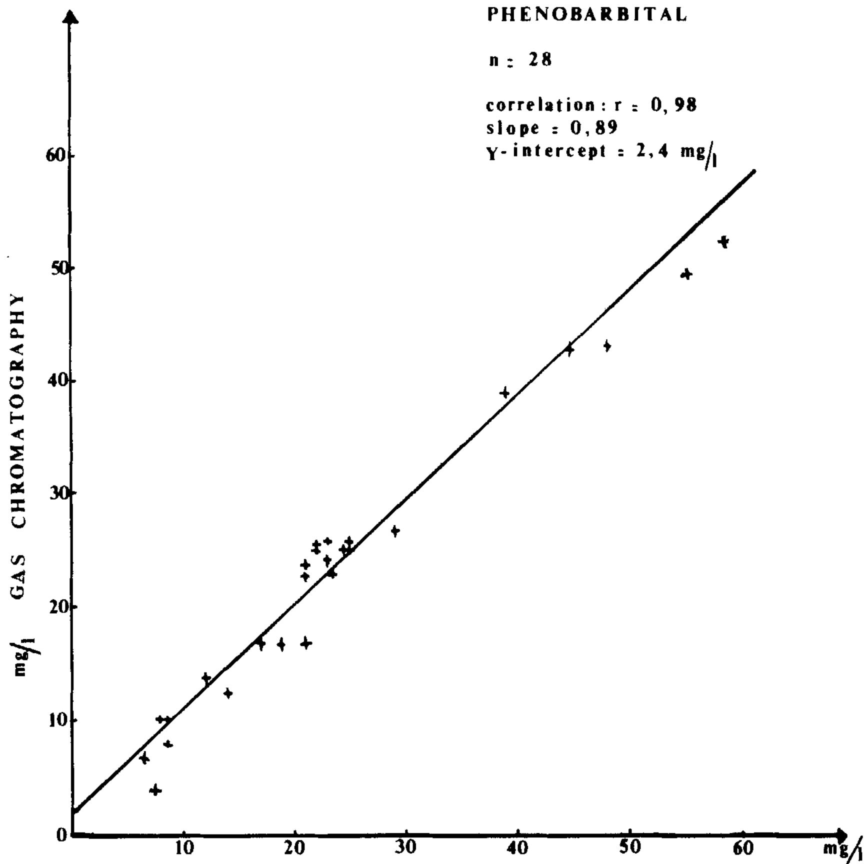

FIGURE 7 Comparison of results obtained for serums analyzed for phenobarbital by HPLC and GC

The results for phenytoin are (n = 17): correlation coefficient 0.99, slope 0.97, y-intercept 0.8 mg/l.

For valproic acid we obtain (n = 15): correlation coefficient 0.98, slope 1, y-intercept 0.6 mg/l.

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