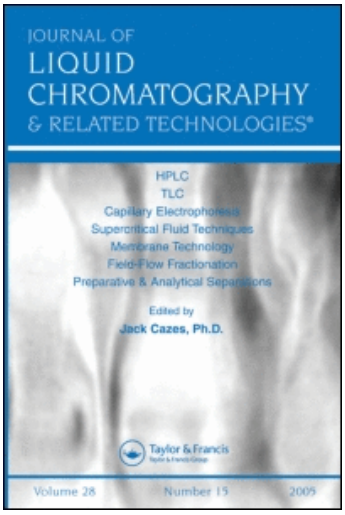

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Abstract: A rapid, precise, and sensitive method has been developed for the simultaneous determination of five antiepileptic drugs (carbamazepine, phenobarbital, diphenylhydantoin, primidone, and oxcarbazepine) and six metabolites (from carbamazepine, carbamazepin-10,11-epoxide; from phenobarbital, *p*-hydroxyphenobarbital; from primidone, 2-phenyl-2-ethylmalonamide; from diphenylhydantoin, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin) in human serum. Separation was achieved by means of liquid chromatography with a diode array detector (DAD) using reverse-phase ODS-Hypersil columns (100×2.1 mm) with particle size of $5\,\mu$m, flow-rate 0.6 mL/minute, column temperature 40°C, wavelength: 210 nm. Mobile phase was a gradient consisting of solvent A: phosphate buffer pH 5.7, solvent B: MeOH. Two procedures were tested for the serum treatment: protein precipitation with acetonitrile and solid-phase extraction with C_{18} cartridges. The best recoveries were found by means of protein precipitation. Solid-phase extraction gave poor recoveries for phenobarbital and its metabolite. The results obtained are suitable in terms of precision, linearity, and accuracy for the simultaneous determination of serum levels of the mentioned drugs in the usual therapeutic ranges.

Keywords: Anticonvulsants, antiepileptic drugs, HPLC

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INTRODUCTION

Epilepsy is one of the more common neurological disorders that affect between 0.5% and 1% of the world population.[1,2] Today, many drugs are available for the treatment of different types of convulsive episodes.

Although the response to clinical treatment is the main indicator for the physician, the control of anticonvulsant serum levels is very useful. Therapy with anticonvulsants is complicated, due to factors inherent to the physiology of each patient. Besides, most of the antiepileptic drugs now in use show a limited safety margin, making it necessary to adjust the doses for the effective and safe use of the drugs.[3,4] Furthermore, a large proportion of patients receive therapy with multiple antiepileptic drugs.[5] Under these circumstances, a reliable treatment must be accompanied by monitoring the levels of blood concentration. Although these determinations are not exact indices of the pharmacological response, they provide more safety in the design of the dosage regime, thus excluding the majority of the individual differences in the dose-effect relationship.

It is well known that the activity of antiepileptic drugs is, in many cases, produced by the metabolites; therefore, their identification and quantification is of interest too, besides those metabolites whose anticonvulsant activity has already been proved. These determinations are very important from the point of view of dosage regimes.

Different methods have been used for the quantification of anticonvulsant levels in biological fluids. HPLC shows some advantages because it allows the simultaneous quantification of several compounds, which cannot be done with the inmunological methods most frequently used in clinical laboratories.[5]

Few HPLC methods have been reported for the simultaneous determination of anticonvulsants.[6–13] Recently, Bugamelli reported the simultaneous determination of six antiepileptic drugs, including oxcarbamazepine and lamotrigine, and two active metabolites.[6]

A fundamental part in the development of an analytical method is sample preparation; both precision and accuracy depend on this step. The methods more usually employed for sample preparation in the analysis of anticonvulsants are: liquid–liquid extraction, protein precipitation, and solid-phase extraction.

The purpose of the present contribution is the development of an HPLC method for the simultaneous quantification of the anticonvulsants more frequently received in our laboratory, namely: carbamazepine (CBZ), oxcarbazepine (OCBZ), phenobarbital (PB), primidone (PRM), diphenylhydantoin (PHT), as well as some of their pharmacologically active metabolites: from carbamazepine, carbamazepin-10,11-epoxide (CBZ-E); from phenobarbital, *p*-hydroxyphenobarbital, (*p*-HPB); from primidone, 2-phenyl-2-ethylmalonamide, (PEMA); from diphenylhydantoin 5-(*p*-hydroxyphenyl)-5-phenylhydantoin, (*p*-HPPH). Two methods for sample preparation are

discussed: protein precipitation with acetonitrile and the solid-phase extraction procedure.

Chemical structures of the drugs under evaluation are shown in Figure 1.

EXPERIMENTAL

Reagents and Solutions

All the solvents employed, including water, were HPLC grade (Merck). The anticonvulsants, carbamazepine, phenobarbital, diphenylhydantoin, and primidone, as well as the metabolites carbamazepin-10, 11-epoxide and 10, 11-dihydro-*trans*-hydroxycarbamazepine (CBZ-diol), *p*-hydroxyphenobarbital, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin, and 2-phenyl-2-ethylmalonamide were from Alltech Applied Science. Oxcarbazepine was supplied by Novartis Pharma. Buffers were prepared with monobasic and dibasic potassium phosphate salts from Sigma. The pHs were adjusted with 0.1 M HCl and NaOH as needed.

A stock solution of each compound was prepared by dissolving the pure compound in methanol (1 mg/mL); the solutions were sealed and stored at 4°C.

As biological material, a pool of human serum samples and individual serum samples from healthy volunteers was used. For the preparation of serum samples containing known amounts of drugs, pooled human drug-free serum was spiked with different volumes from each stock solution, leading to the final concentration range used for each analyte: 2.5–25 μg/mL for *p*-HPB and CBZ; 2.5–15 μg/mL for PRM, *p*-HPPH, CBZ-E and OCBZ; 5–25 μg/mL for PHT and 5–50 μg/mL for PB.

Chromatographic Separation

The HPLC system used was a Hewlett Packard HP-1090 Series II/L with DAD detector and autoinjector. Reverse-phase ODS-Hypersil columns (100 × 2.1 mm) with particle size of 5 μm were used. The mobile phase consisted of: eluent A: 0.1 M phosphate buffer pH 5.7 and eluent B: methanol. All mobile phases were filtered prior to use through a Millipore HVLP membrane filter (0.45 μm). Data were collected and analyzed in an HPLC-3D ChemStation, DOS Series.

Optimization of Separation

Optimization of the analytical separation was achieved by changing flow-rates, temperature and composition of the mobile phase. For each experiment, retention (k'), separation (α) and resolution (R) parameters were calculated.

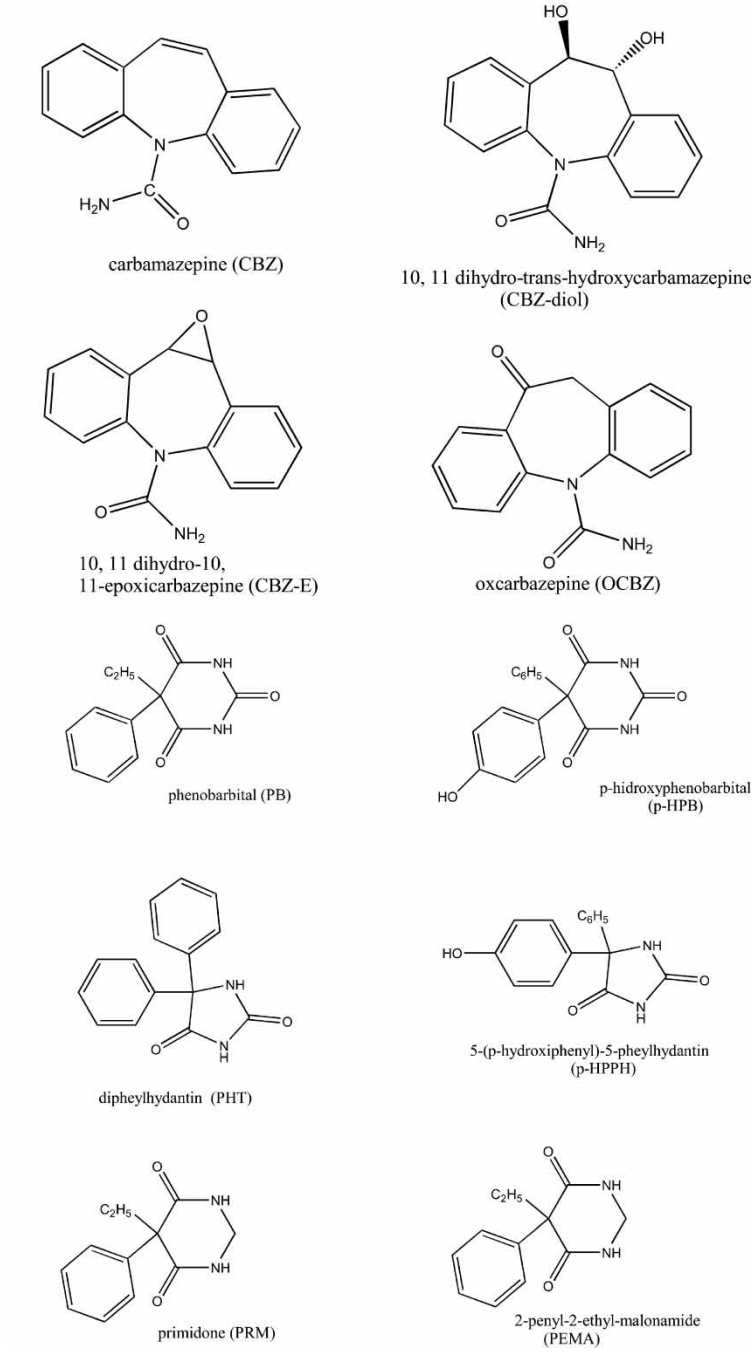

Figure 1. Chemical structures of the anticonvulsants and the metabolites analyzed.

Preparation of Samples

Two procedures were evaluated using control serum samples spiked with each analyte under evaluation, as well as with a mixture of all of them.

Acetonitrile deproteinization: 100 μL of the spiked serum was added to Eppendorf tubes containing 200 μL of acetonitrile; the mixture was vortex-mixed for 10 minutes and then centrifuged at 1500 rpm for 5 minutes. The supernatant was filtered before being injected into the chromatograph.

Solid-phase extraction: Sep-Pak cartridges were employed. They were activated by flushing with 5 mL of acetonitrile, followed by 10 mL of water. After the conditioning step, the spiked serum (0.5 mL) was applied followed by 0.5 mL of phosphate buffer solution pH 5.7. The cartridges were washed with water (20 mL) and the drugs were eluted with a mixture of methanol: acetonitrile (1 : 1.5 mL). 3 The solvent was evaporated under N_2; the sample was resuspended with 1 mL of the mobile phase consisting of MeOH and 0.01 M phosphate buffer pH 5.7 (15 : 85) before being injected into the chromatograph.

Both methods were evaluated using solutions of all the drugs under evaluation at a concentration of 25 μg/mL. Each procedure was repeated five times. Results of precision (RSD) and accuracy (% recovery) were the criteria used for evaluation of the performance.

The optimized method was validated with the following parameters:

Precision

RSD for each analyte was calculated by means of injection of the working standard solutions as well as deproteinized spiked serums. In all cases, the determinations were repeated three times.

Linearity

Five levels of concentration were used. The working interval was chosen by considering the therapeutic range for each drug ± 50%. The resulting mixture was subjected to the sample pre-treatment procedure previously selected and was injected into the chromatograph. The peak area values from each analyte were plotted against the concentration; calibration curves were constructed by means of the least-square method.

Detection and Quantitation Limits

D_L and Q_L were calculated based on the standard deviation of the blank response and the slope of the calibration curves.[14]

$$D_L = Ybl + 3\sigma/s \qquad Q_L = Ybl + 10\sigma/s$$

Where

σ: standard deviation of the blank response

s: slope of the calibration curve

Ybl: estimated blank response (intercept in the calibration curve)

Accuracy

This was evaluated by the % recoveries; they were calculated for each drug at four concentration levels lying within the linearity range previously established; the following relationship was used: 100 (amount recovered/amount added). The assays were repeated three times.

Selectivity

Selectivity was tested by examining the spectroscopic purity level of each peak in the chromatogram obtained from the mixture as compared with the spectroscopic purity level obtained in the chromatogram run for each drug. Matrix interferences were evaluated by analysis of four serum samples from healthy, non-medicated volunteers; in addition, one serum from a patient treated with oxcarbazepine, in which the metabolite 10,11-dihydro-10-hydroxycarbazepine was present, was also evaluated. The possible interference from the metabolite 10,11-*trans*-hydroxycarbazepine was also evaluated by adding it to the standard mixture.

Ruggedness

Variables under consideration were: mobile phase composition, flow-rate, column temperature, and pH.

RESULTS AND DISCUSSION

Separation

Optimized chromatographic conditions were as follows: flow-rate 0.6 mL/minute, column temperature 40°C, wavelength: 210 nm, bandwidth 10 nm. Mobile phase was solvent A: phosphate buffer pH 5.7, solvent B: MeOH. The gradient used for separation is presented in Table 1. The wavelength was chosen after recording the spectra for each compound. Some authors recommend the use of longer wavelengths,[6] the advantage of using the wavelength selected here is that all the analytes under consideration can be quantified simultaneously. Considering that this wavelength is near

Table 1. Elution gradient used for the separation of the anticonvulsants and their metabolites

Time (min)	B %	Delay (min)
0	15	0
0–12	15–45	12
12–18	45	6
18–20	45–15	2

A = phosphate buffer pH = 5.7.
B = methanol.

the cutoff for methanol, spectroscopic grade MeOH was used in order to avoid possible interferences.

A representative chromatogram showing the compounds analyzed is shown in Figure 2. Total time for one analytical run was 18 minutes.

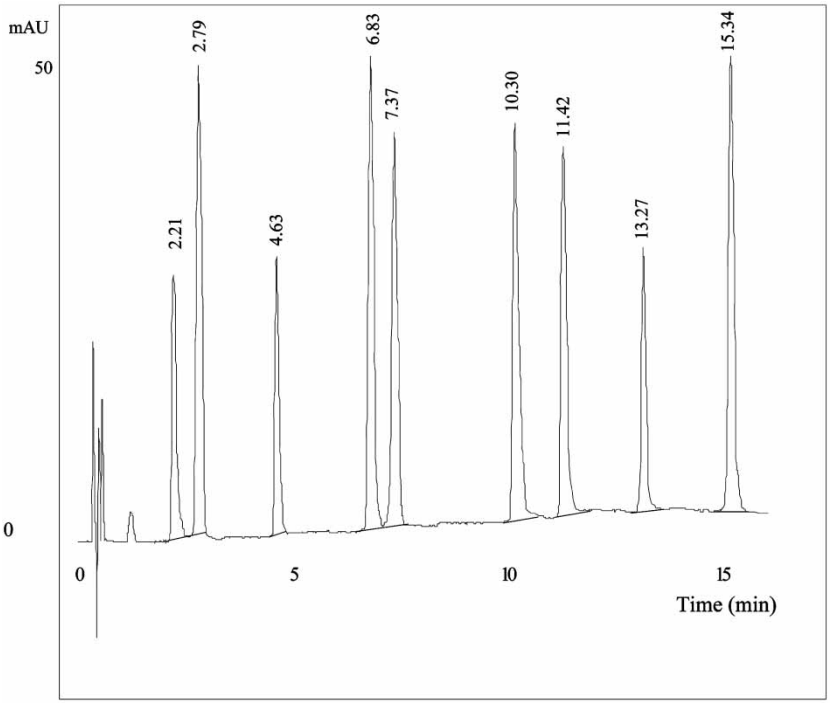

Figure 2. HPLC chromatogram of the anticonvulsants and their metabolites. Elution order is: PEMA, *p*-HPB, PRM, PB, *p*-HPPH, CBZ-E, OCBZ, PHT, CBZ. Separation conditions are described in the text. Abbreviations, see Figure 1.

Optimized chromatographic parameters are given in Table 2. The lower α and R correspond to the pair phenobarbital (PB) and 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH). Taking into account the precisions obtained (both after intra and interday assays), it was considered not necessary to use an internal standard, thus avoiding further interferences.

Preparation of Samples

The importance of the type of matrix selected for the quantification of antiepileptic drugs has been thoroughly discussed in previous papers.[5] Plasma and serum are most frequently used for this purpose. We selected serum as the matrix in order to avoid possible interferences arising from the anticoagulant used.

Percent recovery and precision were used for the evaluation of the two methods employed for serum treatment: solid-phase extraction and acetonitrile precipitation ($n = 3$); the results obtained after the preparation of samples by the two methods are shown in Table 3. For this purpose, only one concentration (25 μg/mL) of all the drugs under evaluation was used. As can be seen in Table 3, %RSD for both methods were satisfactory; however, % recovery was lower with the solid-phase extraction procedure, especially in the case of PB (29% recovery) and its metabolite *p*-HPB, which could not be recovered under the employed conditions; by using acetonitrile precipitation all the analytes could be recovered in good yields. Table 4 shows the % recovery obtained after treatment of the spiked serum with acetonitrile at four concentration levels. The procedure was repeated three times.

Bugamelli et al.[6] report that a deproteinization procedure using perchloric acid was less satisfactory than the use of solid-phase extraction. We employed acetonitrile as a protein precipitation reagent, with precisions comparable to those obtained by means of solid-phase extraction; % recoveries were improved with the deproteinization agent.

Table 2. Chromatographic parameters for separation

Drug	t_R	k'	α	R
PEMA	2.21	3.6	1.27	2.4
p-HPB	2.79	4.6	1.65	5.08
PRM	4.62	7.7	1.47	4.58
PB	6.82	11.3	1.10	1.12
p-HPPH	7.36	12.2	1.30	5.4
CBZ-E	10.30	17.6	1.15	1.80
OCBZ	11.40	19.0	1.16	3.90
PHT	13.29	22.1	1.15	4.27
CBZ	15.34	25.5		

Table 3. Precision and accuracy for the two methods used for serum treatment

	Precipitation with acetonitrile		Solid-phase extraction	
Drug	Recovery (%)	RSD	Recovery (%)	RSD
PEMA	91.0	3.9	59.1	3.5
p-HPB	68.5	12.3	—	—
PRM	101.0	5.5	75.5	1.8
PB	94.0	2.7	29.0	5.6
p-HPPH	92.5	5.3	76.0	3.0
CBZ-E	99.0	4.5	77.8	2.3
OCBZ	93.4	4.6	73.6	5.9
PHT	101.0	3.6	75.0	5.1
CBZ	99.9	6.9	79.5	2.0

$n = 3$.

Linearity

Calibration curves were constructed in the therapeutic concentration ranges for each analyte. Linearity data are reported in Table 5.

Detection and Quantitation Limits

In order to calculate these parameters, calibration curves were constructed at three concentrations levels, one at the lowest point in the calibration range and

Table 4. Validation of parameters: recovery and precision

Drug	Recovery (%)	% RSD
PEMA	82.4–97.2	8.2
p-HPB	62.0–75.0	10.9
PRM	96.6–106.0	4.8
PB	86.8–101.3	6.9
p-HPPH	83.0–102.0	10.5
CBZ-E	88.4–110.0	8.7
OCBZ	89.0–97.8	4.1
PHT	97.6–104.5	7.5
CBZ	94.8–105.0	7.9

*Data are the mean from the results obtained at four concentration levels ($n = 3$ for each level). Sample treatment was by means of protein precipitation with acetonitrile.

Table 5. Linearity parameters for the analytes (protein precipitation)

Drug	Concentration range	R^2	Equation	D_L (μg/mL)	Q_L (μg/mL)
PEMA	2.5–25	0.999	$Y = -0.81 + 0.2X$	1.07	3.38
p-HPB	2.5–15	0.998	$Y = -0.66 + 0.3X$	1.45	2.10
PRM	2.5–15	0.998	$Y = -0.08 + 0.1X$	1.38	1.42
PB	5.0–50	0.999	$Y = -0.44 + 0.2X$	1.60	3.67
p-HPPH	2.5–15	0.995	$Y = -0.67 + 0.3X$	1.50	2.12
CBZ-E	2.5–15	0.995	$Y = -0.83 + 0.3X$	1.45	3.66
OCBZ	2.5–15	0.996	$Y = -0.46 + 0.2X$	0.50	1.00
PHT	5.0–25	0.998	$Y = -0.32 + 0.2X$	1.85	2.82
CBZ	2.5–25	0.998	$Y = -0.90 + 0.3X$	1.98	3.15

Concentration in μg/mL.
Based on peak height.

two points at concentrations lower than this level.[14] The results are presented in Table 5. A number of methods have been proposed for evaluation of D_L and Q_L. The advantage of calculating D_L and Q_L by the method used here is that matrix effects are taken into consideration.

Selectivity

Spectroscopic purity was determined for each analyte in the 200–400 nm interval. Purity levels obtained ranged from 92.1 for the epoxide from carbamazepine (retention time 9.4 minutes) to 99.9 for PHT (retention time 14.1 minutes) The inactive metabolite from carbamazepine: carbamazepine-10,11-dihydroxide appeared at 8.1 minutes, showing no interferences with any of the eluted drugs. The chromatogram of one serum obtained from a patient under oxcarbamazepine treatment, presented a major peak at 8.7 minutes; it was assumed that this peak corresponds to 10,11-dihydroxyoxcarbazepine,[12] no interference was seen between this peak and any of the drugs under evaluation.

Ruggedness

Factors evaluated were: changes in mobile phase (the first 12 minutes from the gradient from 10% to 60% MeOH); in pH (5 and 6 units); temperature (35 and 45°C) and flow rate (0.5 and 0.7 mL/min). Although some variations in retention times could be seen, no loss in resolution or elution order was observed in all the chromatographic runs. Matrix effects were tested by

means of the analysis of eight control human sera from different healthy individuals added to the analytes. No interferences were observed arising from the matrix.

CONCLUSIONS

A highly selective method has been developed for the simultaneous determination of five anticonvulsants and their active metabolites in serum. In the present case, protein precipitation with acetonitrile gave better results than solid-phase extraction in terms of % recovery. The sensitivity and linearity obtained are adequate for use in monitoring anticonvulsant levels, as they are within the established therapeutic range for each drug analyzed. The method showed stability under the modifications made in the ruggedness experiments. No matrix interferences were seen, in spite of the wavelength used.

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