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THIN-LAYER CHROMATOGRAPHIC METHOD FOR DETERMINING CARBAMAZEPINE AND TWO OF ITS METABOLITES IN SERUM

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SUMMARY

A sensitive and highly specific thin-layer chromatographic method for determining simultaneously serum levels of carbamazepine and two of its major metabolites, carbamazepine-10,11-epoxide and 10,11-dihydroxycarbamazepine, is presented. Serum (1 μl) was spotted directly onto the thin-layer plate and, after irrigation, the separated spots were converted into fluorescing compounds by exposing the plates to hydrogen chloride gas for 5 min and then to strong ultraviolet radiation from a mercury lamp for 20 min. The fluorescence was measured quantitatively using a spectrofluorimeter equipped with a thin-layer chromatogram scanning attachment. Two microlitres of serum are sufficient for a duplicate determination.

INTRODUCTION

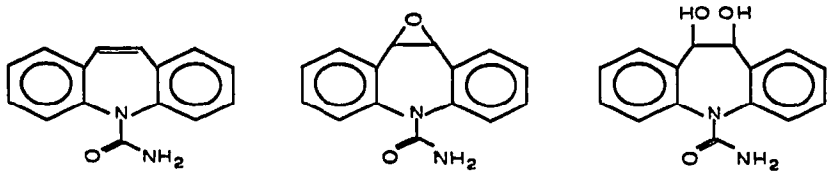
The initiation of a clinical trial with carbamazepine [5-carbamyl-5H-dibenzo-(*b,f*)azepine] in order to compare it as an anticonvulsant with phenobarbitone and diphenylhydantoin necessitated a sensitive and specific method for its determination in order to determine the correlation between serum levels of carbamazepine and the clinical response in treated patients. In recent years, much attention has been paid to the problem of determining carbamazepine in body fluids and several methods involving gas–liquid chromatography[1–7], spectrophotometry[8,9], thin-layer chromatography (TLC)[10,11] and combined gas chromatography–mass spectrometry[12] have been reported. All these methods employed some procedure for extraction from serum or plasma volumes of not less than 0.5 ml.

This paper describes a TLC method by means of which carbamazepine and two of its metabolites, carbamazepine-10,11-epoxide and 10,11-dihydroxycarbamazepine, can be determined simultaneously, only 2 μl of serum being required for a duplicate determination and no extraction procedure being involved.

EXPERIMENTAL

Reagents

The chemical structures of the compounds investigated are shown in Fig. 1.



I II III

Fig. 1. Chemical structures of carbamazepine (I), carbamazepine-10,11-epoxide (II) and 10,11-dihydroxycarbamazepine (III).

Carbamazepine (I) was obtained from Ciba-Geigy, Johannesburg, South Africa. Carbamazepine-10,11-epoxide (II) and 10,11-dihydroxycarbamazepine (III) were obtained from Ciba-Geigy, Basle, Switzerland. Stock solutions were made up in absolute methanol and stored at −20°. Benzene, ethyl acetate and methanol were guaranteed reagent grade (Merck, Darmstadt, G.F.R.).

Apparatus

A Perkin-Elmer MPF3 spectrofluorimeter equipped with a thin-layer chromatogram scanning attachment was used to measure the fluorescence of the spots on the thin-layer plates using the following operating conditions: light source, xenon lamp; excitation wavelength, 354 nm; emission wavelength, 486 nm; emission filter, 430 nm; excitation slit width, 10 nm; scanning speed, high (4 cm/min); chart paper speed, low (2.5 cm/min). The emission slit width, amplifier sensitivity, zero suppression and sample adjustment were set so as to obtain approximately 80% full-scale deflection on the recorder when the strongest spot in the chromatogram was being scanned. For peaks smaller than 20 mm in height in the same chromatogram, the amplifier sensitivity, zero suppression and sample adjustment were re-set but the emission and excitation slit widths were left unaltered. Typical settings for obtaining 80% full-scale deflection for a spot containing 16 ng of carbamazepine were: amplifier sensitivity, 0.3; emission slit width, 6 nm.

The other apparatus used consisted of silica gel 60 TLC plates, dimensions 10 × 20 cm (Merck), a Hamilton dosing syringe (10 μl), 5-μl disposable glass capillary micropipettes calibrated at 1-μl intervals (Clay Adams Division of Becton, Dickinson & Co., Parsippany, N. J., U.S.A.), and a Universal UV lamp, type TL-900/U (Camag, Muttenz, Switzerland).

Stock solutions

A single stock solution containing 10.00 mg of I, 2.50 mg of II and 2.50 mg of III in 10 ml of absolute methanol was prepared as follows. The reagents were weighed separately on a Mettler ME 22 electronic microbalance and solutions made up with methanol to contain 30.00 mg of I, 7.50 mg of II and 7.50 mg of III per 10 ml of solution by weighing the requisite amounts of methanol into the sample tubes. Equal amounts of these solutions were then added together so as to give the final stock solution, which was stored at −20°. (This procedure was necessary because of the limited amounts of metabolites available.)

Standard solutions

Four standard solutions were prepared by adding, with a Hamilton micro-

syringe, 2, 4, 8 and 16 μl of the stock solution in each instance to 1 ml of fresh human serum contained in plastic-capped sample tubes. These standard solutions were kept frozen at —20° when not in use and were found to be usable for at least 4 weeks. The weight of the stock solution was carefully monitored after each preparation of standard solutions and weight losses due to evaporation were corrected for by adding the requisite weight of methanol to it.

Spotting the plates

Exactly 2 μl water was taken up in a calibrated, disposable micropipette followed by exactly 1 μl of the serum to be investigated or a standard serum solution. The whole 3 μl of solution were then spotted on the plate in a single, smooth application. In this fashion, sixteen spots with unknown serum and standard serum alternating in duplicate were applied to a single 10 × 20 cm plate. This corresponds to four unknown serum samples being processed in duplicate.

Chromatography

The eluent used was ethyl acetate–benzene–methanol (5:4:1). The development was carried out in an unsaturated tank up to a height of 9 cm; the elution time was *ca.* 20 min. After drying them briefly with a hair-dryer, the plates were dried in an oven at 100° for 5 min and then exposed for 5 min to hydrogen chloride gas generated in a chromatographic tank by pipetting 2 ml of concentrated hydrochloric acid into 10 ml of concentrated sulphuric acid contained in a small beaker in the tank. Immediately after exposure to hydrogen chloride gas, the plates were irradiated for 15 min with ultraviolet light obtained from an unfiltered mercury lamp. This procedure converted the carbamazepine and the two metabolites into fluorescent compounds that could be measured directly and quantitatively with the TLC scanning attachment of the MPF3 spectrofluorimeter. Each series of spots corresponding to an applied sample was scanned in the direction of the solvent flow. Standard curves were constructed by plotting peak heights *versus* the serum concentrations of the known standards. The concentration of an unknown sample was then obtained by interpolation.

RESULTS AND DISCUSSION

Fig. 2 represents part of a densitogram showing the peaks obtained for standard serum containing the indicated amounts of I, II and III as well as sera from two different patients.

Typical standard graphs of peak heights *versus* serum concentrations of I, II and III are shown in Fig. 3. From these graphs, it appeared that over the range investigated a linear relationship existed between the concentration of carbamazepine and peak height. The same could not be claimed for the two metabolites, although the epoxide gave a much closer approximation to a linear relationship than 10,11-dihydroxycarbamazepine.

From the peak heights obtained for the known concentrations of carbamazepine, an equation of the best straight-line fit was obtained by linear regression analysis, and this equation was used to calculate the concentrations of carbamazepine in unknown sera from the peak heights measured. The concentrations of the metabolites

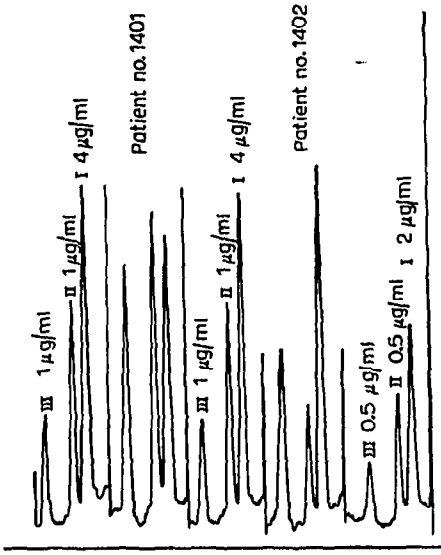


Fig. 2. Densitogram of different amounts of carbamazepine and the two metabolites in serum.

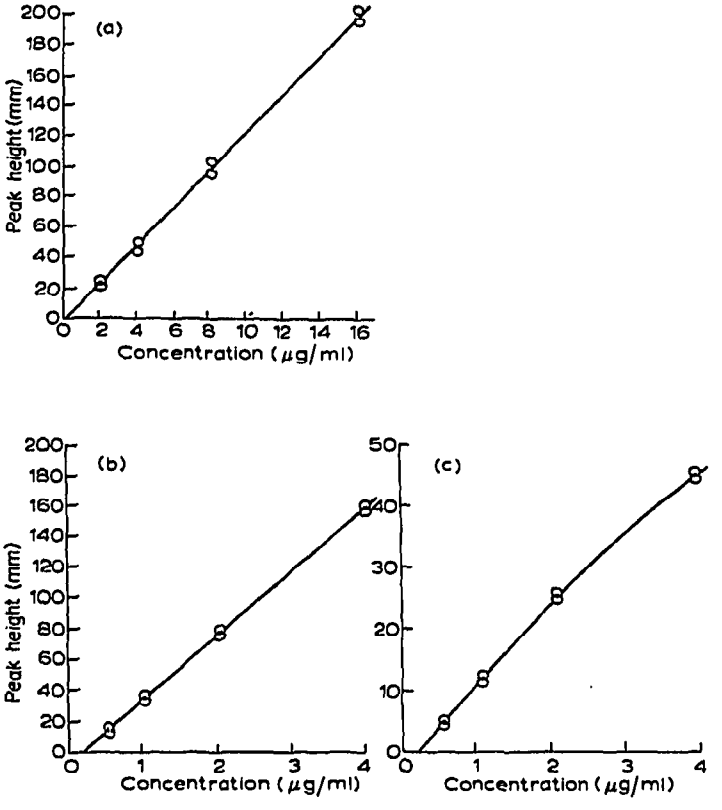


Fig. 3. Calibration curves for (a) carbamazepine, (b) carbamazepine-10,11-epoxide and (c) 10,11-dihydroxycarbamazepine in serum.

TABLE I

RECOVERY OF COMPOUNDS INVESTIGATED

Compound	*Amount added (μg/ml)*	*Amount found (μg/ml)*				*Mean ± S.D. (μg/ml)*
Carbamazepine	3	2.96	3.32	3.10	2.65	3.01 ± 0.28
	6	5.96	6.04	5.80	5.72	5.88 ± 0.15
	11	11.45	10.70	11.28	11.90	11.33 ± 0.50
	14	14.20	14.16	12.96	13.62	13.74 ± 0.58
Carbamazepine-10,11-epoxide	0.75	0.74	0.84	0.77	0.75	0.78 ± 0.05
	1.50	1.44	1.45	1.42	1.44	1.44 ± 0.01
	2.75	2.84	2.74	2.77	2.92	2.82 ± 0.08
	3.50	3.57	3.41	3.16	3.63	3.44 ± 0.21
10,11-Dihydroxycarbamazepine	0.75	0.72	0.85	0.72	0.66	0.74 ± 0.08
	1.50	1.59	1.44	1.44	1.46	1.48 ± 0.07
	2.75	2.82	2.75	3.08	2.90	2.89 ± 0.14
	3.50	3.39	3.17	3.41	3.26	3.30 ± 0.11

in unknown sera were obtained by interpolation from standard graphs of peak heights *versus* serum concentrations of metabolites.

A summary of the recoveries of I, II and III added to human sera within the ranges 2–16 μg/ml for I and 0.5–4 μg/ml for II and III is presented in Table I.

The lowest demonstrable concentration of carbamazepine in serum was about 0.1 μg/ml, while those of each of the metabolites corresponded to about 0.05 μg/ml. Fig. 4 represents a densitogram obtained for standard serum samples at very low concentrations of I, II and III.

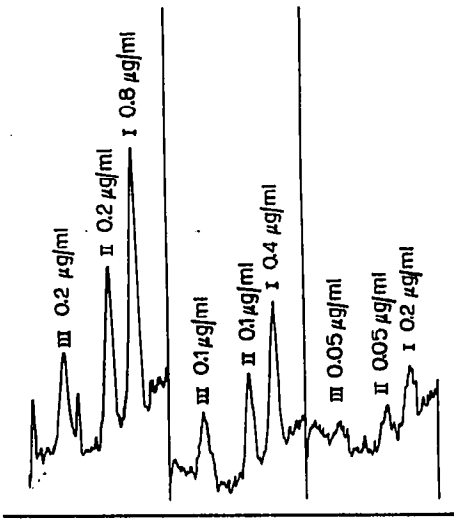


Fig. 4. Densitogram of very low concentrations of carbamazepine and the two metabolites in serum.

Specificity of the method

Sera of a large number of patients receiving a diversity of drugs were chromatographed and treated as described above and in no instance was any spot found that could possibly interfere in the assay for carbamazepine or the two metabolites investigated. The R_F values of I, II and III were found to be 0.49, 0.41 and 0.23, respectively.

The reproducibility is good and the method is sufficiently sensitive and specific for therapy control purposes. The precision could probably be improved by using a more sophisticated spotting device and by a ten-fold dilution of the stock solution so that larger volumes could be pipetted with more accuracy into sample tubes, the solvent evaporated off, and the residue then re-dissolved in 1 ml of serum so as to obtain the standard serum solutions. The fact that no extraction is required gives advantages that need not be emphasized. The very small amounts of sample required for the assay make the method ideally suitable for the study of carbamazepine and the two metabolites in body fluids that usually can be obtained only in small amounts (*e.g.*, cerebrospinal fluid).

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