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RAPID THIN-LAYER CHROMATOGRAPHIC METHOD FOR THE SIMULTANEOUS DETERMINATION OF CARBAMAZEPINE, DIPHENYLHYDANTOIN, MEPHENYTOIN, PHENOBARBITAL AND PRIMIDONE IN SERUM

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SUMMARY

A thin-layer chromatographic method for the simultaneous determination of five anticonvulsant drugs is presented. The serum is extracted with toluene and the dried extract is dissolved in chloroform and applied on to a thin-layer chromatographic plate. After development, the plate is scanned at 215 nm without staining. The drug peaks are well defined. Most of the interfering substances that occur naturally in serum are soluble in and eliminated by the liquid front.

INTRODUCTION

The extraction procedure used in this work is the same as that used by Solow et al. [1] in their gas—liquid chromatographic (GLC) method. We have avoided the complicated staining techniques used in other quantitative thin-layer chromatographic (TLC) methods [2, 3] by measuring directly the diffuse light reflectance from the native drugs with a Zeiss chromatogram spectrophotometer at 215 nm.

EXPERIMENTAL

Apparatus

A BTL shaker (Baird & Tatlock, London, Great Britain), a Hamilton 50-μl precision syringe and a Zeiss chromatogram spectrophotometer were used.

Materials

Reagent-grade sodium dihydrogen orthophosphate, toluene, chloroform, acetone and absolute ethanol were obtained from Merck (Darmstadt, G.F.R.).

Pre-coated silica gel 60 TLC plates (without fluorescent indicator), 20 × 20 cm with a layer thickness of 0.25 mm, were also obtained from Merck.

A working standard solution of the native drugs caffeine, carbamazepine (Tegretol, donated by Ciba-Geigy, Basle, Switzerland), diphenylhydantoin, mephenytoin (Mesantoin, donated by Sandoz, Basle, Switzerland), phenobarbital and primidone (donated by Imperial Chemical Industries, Macclesfield, Great Britain) in absolute ethanol at a concentration of 25 mg per 100 ml of each drug was prepared. This solution is stable for at least 6 months at room temperature.

Procedure

A 300-µl sample of serum is placed in a 12-ml Sovirel screw-topped tube and 150 µl of 0.3 *M* NaH_2PO_4 solution and 5 ml of toluene are added. The tube is tightly closed and shaken vigorously for 3 min with a BTL shaker. The tube is then centrifuged for 10 min at 1400 *g*. A 4-ml volume of the toluene phase is transferred into a centrifuge tube, placed in a 60° water-bath and evaporated to dryness by means of a direct air stream. The residue is dissolved in 50 µl of chloroform and the solution obtained is applied with a Hamilton syringe as a very thin streak on a TLC plate at a width of 0.8 cm and along an axis 1.5 cm from the bottom. The TLC plate is placed in an unlined glass chromatography tank containing 100 ml of chloroform—acetone (85:15). Approximately 1.5 h are required for the development of the plate.

A standard curve is prepared by adding 5, 10, 20, 30 and 40 µl of the working standard solution to drug-free serum. The highest point in this curve corresponds to a drug level of 33 µg/ml in serum, which covers the therapeutic range of these drugs (samples with higher values are diluted with drug-free serum). The TLC plate is scanned at 215 nm for diffuse reflectance by means

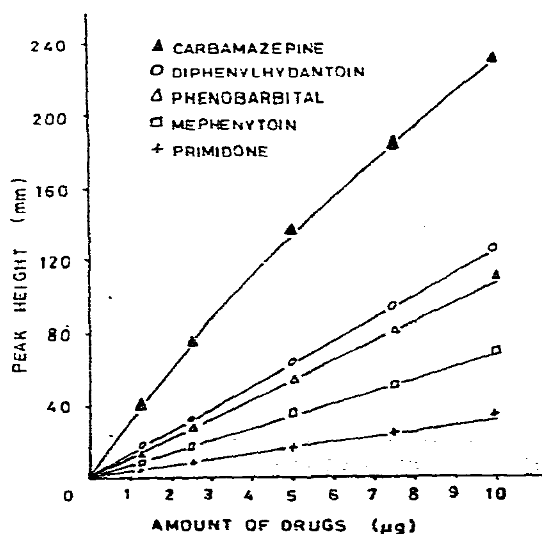


Fig. 1. Standard curves for the drugs extracted from serum, expressed as peak height versus amount.

of a Zeiss chromatogram-spectrophotometer. The result obtained from the standard curve (Fig. 1) is multiplied by 3.33 in order to calculate the concentration in microlitres per millilitre of serum.

RESULTS AND DISCUSSION

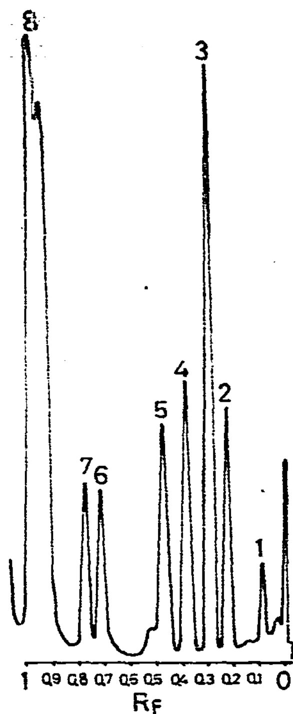
One of the advantages of our TLC method compared with most GLC methods is that derivatization is unnecessary. Some GLC assays have been described that do not require derivatization [4, 5], but in these assays diphenylhydantoin and phenobarbital give asymmetric peaks and a decrease in sensitivity. It has also been demonstrated that unmethylated diphenylhydantoin tends to be adsorbed on the support [6]. Another advantage of the TLC method over GLC methods is the possibility of scanning the spots on the TLC plate directly in the UV range in order to obtain the absorption spectrum and then comparing this pattern with known absorption spectra for positive identification. In the TLC method, time is employed more efficiently than in GLC methods: there is approximately 1.5 h of free time during the development of a TLC plate, compared with 20 min of waiting for a GLC run with only four of our drugs [7]. Our method has proved to be faster with a greater volume of tests, even when the time of application (1.5 min) and scanning (1.5 min) per test is taken into consideration.

The chromatographic separation shown in Fig. 2 was carried out at room temperature (20—25°). It was found that when the tank was not lined with paper, the time for chromatography increased from 60 to 90 min, but the separation pattern of the drugs was improved.

As shown in Fig. 3, the wavelength of 215 nm selected does not correspond to the absorption maxima of drugs tested. This wavelength was chosen because below 215 nm the high attenuation gave an unsteady baseline and in the range 270—300 nm, where caffeine and carbamazepine have their maximal absorption, the other drugs exhibit no absorption. Serum extracts from many of our patients showed a peak at R_F=0.22, which was found to be caffeine and was therefore included in our working standard (Fig. 2).

In our acid extraction method we used a dilution technique which maintains a constant buffered system by retaining the total serum volume. We found this to be necessary when diluting serum containing carbamazepine, which is usually extracted from an alkaline medium, in order to maintain a serum buffered system comparable with that employed for the standard curve. This precaution is not necessary when diluting the other drugs, but is used to lend uniformity to our procedure.

In over 1 year of using the method daily, we have observed few interferences One interferent, however, is ethylphenacemide, which has the same R_F value as diphenylhydantoin. These substances can be differentiated by using toluene—acetone (80:20). Another interferent is the antibiotic Bactrim, which is composed of sulfamethoxazol and trimethoprim; in this instance sulfamethoxazol has the same R_F value as carbamazepine. Differentiation between these two substances was accomplished with an alkaline extraction procedure. In cases of patients taking mephenytoin, demethylated mephenytoin [8] has the same R_F value as carbamazepine. This presents no problem, however, as



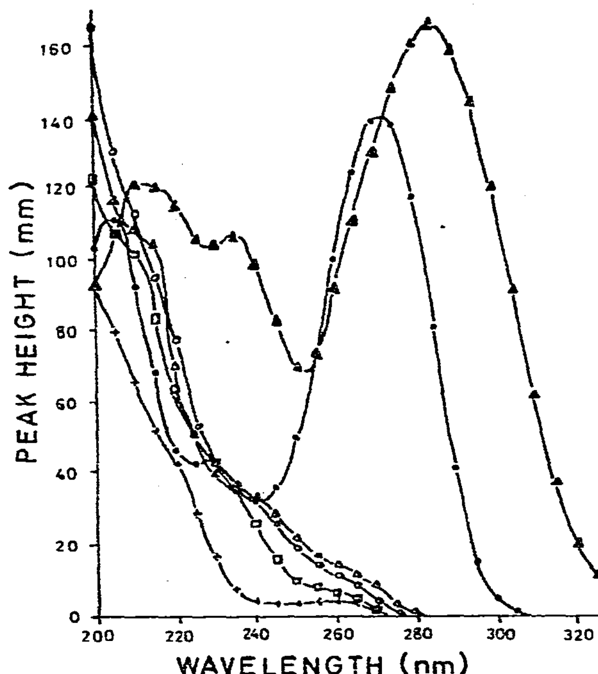


Fig. 2. Results obtained from a scan at 215 nm of a TLC plate after the separation of a serum containing 16.5 μg/ml each of primidone (1), caffeine (2), carbamazepine (3), diphenylhydantoin (4), phenobarbital (5) and mephenytoin (6). Peak 7 is an unidentified serum peak and peak 8 is the solution front of lipids.

Fig. 3. Ultraviolet absorption spectrum obtained by scanning a TLC plate containing 2.5 μg each of caffeine (•) and carbamazepine (▲) and 5.0 μg each of diphenylhydantoin (○), mephenytoin (□), phenobarbital (△) and primidone (+), which were applied directly on to the plate, chromatographically separated and measured in situ by means of a Zeiss chromatogram-spectrophotometer.

these two substances are not been taken together. As the metabolite of mephenytoin exhibits the same UV absorption curve as mephenytoin, it is easily distinguished from carbamazepine.

The recovery and reproducibility of the method are presented in Table I. The recoveries were obtained by comparing the drugs directly applied in the same amounts as the drugs being extracted from serum, applied and separated. The reproducibility is the result of 30 analyses of the same serum sample to which the five drugs were added.

The reproducibility indicated in Table I is better than that given by Simon et al.'s TLC method [9], with which 30 samples of diphenylhydantoin gave a mean value of 23.0 μg/ml with a standard deviation of ± 2.07 μg/ml, which gives a coefficient of variation of 9.0%. On comparison with the GLC method of Grimmer et al. [10], which gave a coefficient of variation of 4.5% for diphenylhydantoin, and the Emit® (Palo Alto, Calif., U.S.A.) system, which

TABLE I

RECOVERY OF DRUGS FROM SERUM AND REPRODUCIBILITY OF THE METHOD

Drug	Recovery (%)	Reproducibility (30 samples)	
		Mean ± S.D. (µg/ml)	C.V. (%)
Carbamazepine	99.0	12.6 ± 0.5	4.3
Diphenylhydantoin	90.2	11.9 ± 0.6	4.7
Mephenytoin	79.3	13.7 ± 0.8	5.9
Phenobarbital	86.0	13.0 ± 0.8	6.0
Primidone	51.0	13.3 ± 0.7	5.2

gave a coefficient of variation with phenobarbital as high as 15.0%, we conclude that our quantitative TLC method is precise and rapid and our results are well within the accepted limits of deviation.

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