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RAPID MICROANALYSIS OF ANTICONVULSANTS BY HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A rapid microanalytical method is described for phenobarbital, phenytoin, primidone and carbamazepine utilizing high-performance thin-layer chromatography (HPTLC). This procedure incorporates a single extraction of a 50-μl plasma sample. One tenth of the extract is concentrated and applied to the HPTLC plate by a Contact Spotter, chromatographically separated and quantitated by in situ ultraviolet reflectance densitometry. The coefficient of variation is less than 4% (n = 8), the extraction efficiency is approximately 95% and the minimum detectable amount of pure drug standards applied to and developed on the HPTLC plate is 5 ng or less for all four anticonvulsants.

INTRODUCTION

A variety of methods have been reported for the determination of anticonvulsant drugs in blood plasma which, with the exception of immunoassay techniques [1], are for the most part based on gas [2] or liquid [3] chromatographic separations. Column chromatographic procedures are usually quite adequate where the number of samples to be examined is modest and the analysis time is not critical. However, if these factors are an important consideration, the ability to process many samples simultaneously becomes a definite advantage. It is for this reason that thin-layer chromatography (TLC) with in situ densitometric quantitation is an attractive alternative to column chromatography, particularly where many samples must be assayed in a short period of time.

Methods utilizing TLC in blood level determinations have been described for most of the important anticonvulsant drugs [4—8]. High-performance thin-layer chromatography (HPTLC), however, permits even greater speed of

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analysis than realized with conventional TLC [9], and perhaps more importantly offers significant improvement in sensitivity of detection [10]. It is this latter feature which has led to the development of this microanalytical method for the determination of phenobarbital, phenytoin, primidone, and carbamazepine in blood plasma.

This procedure offers many important advantages over existing procedures [4—8]. It incorporates a single, rapid extraction of a 50-μl plasma sample, fifteen extracts may be concentrated and applied simultaneously to the HPTLC plate by means of contact spotting [11], and after development the drugs are measured by in situ ultraviolet (UV) reflectance densitometry. The method lends itself to the analysis of large numbers of samples as well as to emergency situations where a rapid and accurate assay is required.

MATERIALS AND METHODS

Apparatus

Samples were extracted in 1.5-ml Eppendorf polypropylene test tubes (Brinkmann Instruments, Westbury, NY, U.S.A.). HPTLC plates (silica gel 60 F254, E. Merck, Darmstadt, G.F.R.) were pre-cleaned by overnight development in absolute ethanol in the presence of ammonia vapor. A Shimadzu Model C-910 dual-wavelength TLC scanner (Shimadzu Scientific Instruments, Columbia, MD, U.S.A.) was used for densitometric determinations, and samples were applied to the HPTLC plates with a Contact Spotter (Clarke Analytical Systems, Sierra Madre, CA, U.S.A.). All glassware was silylated by a vapor phase method [12].

Reagents

Drugs used in preparation of standards were phenobarbital (Merck, Rahway, NJ, U.S.A.), phenytoin (Aldrich, Milwaukee, WI, U.S.A.), primidone (Ayerst Labs., New York, NY, U.S.A.), and carbamazepine (Ciba Pharmaceuticals, Summit, NJ, U.S.A.). The internal standard was *p*-tolylbarbital (Aldrich Chemical).

Chloroform, isopropanol, and ammonium hydroxide were ACS grade and were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Absolute ethanol was from U.S. Industrial Chemicals (New York, NY, U.S.A.).

Preparation of standard solutions and plasma

Dissolve each drug in absolute alcohol to provide stock solutions of the following concentrations: phenobarbital, 1.0 g/l; phenytoin, 1.0 g/l; primidone, 4.0 g/l; carbamazepine, 0.20 g/l; and *p*-tolylbarbital (internal standard), 1.0 g/l. Introduce 0.5-, 1.5-, and 2.5-ml volumes of each stock solution (with the exception of the internal standard) into three 50-ml volumetric flasks. Evaporate the solvent with gentle heating under a flow of nitrogen and then fill to volume with drug-free plasma. Mix for 2 h, pipette 0.5-ml volumes into 1.5-ml polypropylene test-tubes and freeze for future use. These standards contain the following concentrations of phenobarbital, phenytoin, primidone, and

carbamazepine for the 0.5-, 1.5-, and 2.5-ml original volumes respectively: 10, 10, 4 and 2 mg/l; 30, 30, 12, 6 mg/l; and 50, 50, 20 and 10 mg/l.

Prepare a spotting standard by diluting 50 μl of each drug stock solution and 25 μl of the internal standard solution to 10 ml in a volumetric flask with ethyl acetate containing 0.01% octanol. The concentration of drugs in this solution is equivalent to the central points of the calibration curves produced by extracts of the plasma standards and thus serves as a external standard to monitor the extraction procedure.

Dilute 250 μl of the internal standard stock solution to 100 ml in ethyl acetate. This will be used as the plasma extraction solvent.

Procedure

Pipette 50 μl of the plasma standards and the patient plasma into 1.5-ml polypropylene test-tubes and add 300 μl of ethyl acetate containing the internal standard to each. Vortex the contents of the tubes for 1 min, allow to stand for an additional 10 min, and centrifuge for 5 min at 1000 *g*. Remove 30-μl aliquots of the upper solvent layers and deposit these in the concave sample indentations of the Contact Spotter alongside a 30-μl volume of the spotting standard. Evaporate under nitrogen flow at the low temperature setting. Transfer the residues to a 5 × 10 cm pre-washed HPTLC plate.

Develop the plate in a pre-equilibrated filter-paper lined chamber containing chloroform, together with a separate beaker of concentrated ammonium hydroxide. This solvent system moves the carbamazepine to an R_F of 0.4 and leaves the remaining anticonvulsants at the origin. Measure the carbamazepine by reflectance densitometry at 285 nm wavelength.

Re-develop the plate in chloroform—isopropanol—ammonium hydroxide (80:20:1), again in a pre-equilibrated tank with ammonium hydroxide present in a separate beaker. Two 7-min developments are usually required for complete separation. Scan the developed plate in the dual wavelength mode using 285 nm as the reference and 215 nm as the sample wavelengths.

RESULTS AND DISCUSSION

The in situ UV absorption spectra of the four anticonvulsants are shown in Fig. 1. At 285 nm no other drugs, metabolites or components from plasma extract of patients have been found which interfere with the detection and measurement of carbamazepine. Dual wavelength measurement for the remaining anticonvulsants is preferred over a single wavelength because of the greater signal gain required at 215 nm. Baseline noise due to surface irregularities in the HPTLC plate, light source variations, etc., are reduced significantly by compensation with a reference wavelength. As is the case with carbamazepine, no components have been found to interfere with the measurement of phenobarbital, phenytoin, primidone, and the internal standard using this system.

Fig. 2 shows the HPTLC separation of a plasma extract containing carbamazepine scanned at 285 nm and the remaining drugs scanned at 215 nm with the reference wavelength at 285 nm. The standard curves of the peak height ratios of the four drugs to the internal standard at concentrations

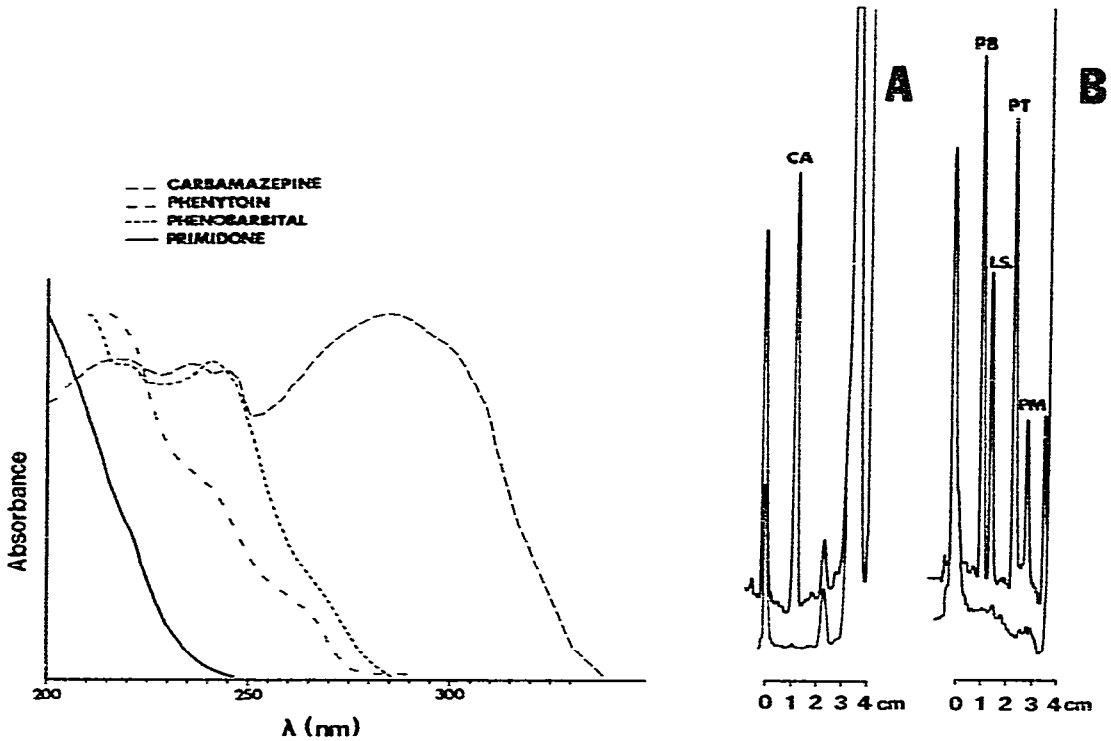

Fig. 1. UV absorption spectra obtained by scanning a HPTLC plate on which the four anticonvulsants had been spotted and chromatographically separated.

Fig. 2. (A) Chromatographic separation after first development system of a plasma extract containing 5 mg/l of carbamazepine (CA) and a blank plasma extract; HPTLC plate was scanned by reflectance densitometry at 285 nm. (B) Chromatographic separation after second solvent system of plasma extract containing 40 mg/l of phenobarbital (PB), *p*-tolylbarbital (internal standard, I.S.), 20 mg/l of phenytoin (PT), and 10 mg/l of primidone (PM) and a blank plasma extract; plate scanned by dual wavelength reflectance densitometry (285 nm reference and 215 nm sample wavelengths).

covering the therapeutic ranges are shown in Fig. 3. Each point represents the average of three determinations, and in each case the difference between values and the mean is less than 3%. Within-run precision obtained by processing eight aliquots of a single plasma sample through the complete procedure is shown in Table I. The extraction efficiency for all four anticonvulsants is greater than 95%, and minimum detectable amounts of pure drug standards applied to the HPTLC plates, developed, and scanned are approximately: phenobarbital, 5 ng; phenytoin, 3 ng; primidone, 3 ng; and carbamazepine 1 ng. With the extraction of 50 μl of plasma the minimum detectable amount is 2, 2, 2, and 0.5 μg/ml for phenobarbital, phenytoin, primidone and carbamazepine respectively. If greater sensitivity is required a larger amount of plasma may be used or a larger amount of the ethyl acetate extract may be placed on the HPTLC plate.

HPTLC plates containing fluorescent indicator are used in this procedure, because, aside from the obvious advantage of permitting observation of the development process, separation between phenobarbital and the internal

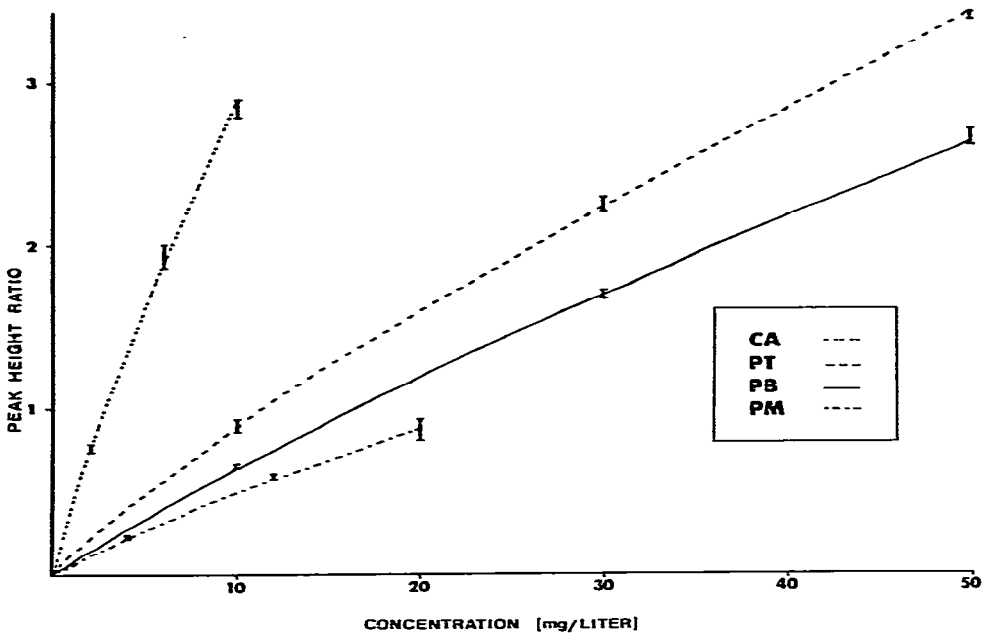

Fig. 3. Calibration curves of carbamazepine (CA), phenytoin (PT), phenobarbital (PB), and primidone (PM) expressed as the peak height ratio to the internal standard as a function of plasma concentration.

TABLE I

REPLICATE DETERMINATIONS OF ANTICONVULSANTS IN PLASMA

Replicate determination of a single plasma sample containing phenobarbital (PB), phenytoin (PT), primidone (PM), and carbamazepine (CA).

No.	PB	PT	PM	CA
1	31.5	32.0	13.4	6.3
2	30.1	31.3	13.0	5.8
3	29.7	31.3	13.2	5.8
4	29.7	31.2	13.4	6.0
5	29.0	31.0	13.4	6.2
6	29.2	30.3	13.7	6.1
7	28.8	30.3	13.2	6.1
8	29.4	29.2	12.0	5.8
$\bar{X}$	29.7	30.8	13.2	6.0
S.D.	0.85	0.68	0.51	0.20
CV (%)	2.9	2.8	3.9	3.3

standard is somewhat improved over that of non-fluorescent plates. Occasionally these compounds do not achieve baseline separation, but this problem can be remedied by adjusting the amount of ammonium hydroxide in the developing solvent or by developing one additional time in the final solvent system.

Clean HPTLC plates are of course quite important for successful determinations at the relatively short wavelengths at which phenobarbital, phenytoin,

and primidone are measured. It is therefore necessary to clean the plates of any components adsorbed from the atmosphere or from packaging materials prior to use. This is easily accomplished by developing the plates overnight in ethanol in an ammonia atmosphere. After this treatment the plates may be stored in a sealed glass container until needed. Development solvents were used as received without contributing any significant contamination.

A single plasma sample with a standard curve can be extracted, chromatographed and quantitated within 2 h. A much larger number of samples can be processed in approximately the same period of time since the extraction and chromatography are carried out concurrently. The densitometric scanning and quantitation is a sequential process which requires approximately one additional minute per sample.

It should be noted that even with a plasma volume of 50 μl, only one-tenth of the sample extract is actually used for a determination. Thus, with some refinement of the method, it should be entirely feasible to utilize blood samples collected from capillary sources, an important consideration with patients where venipuncture may be difficult.

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