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SIMULTANEOUS GAS CHROMATOGRAPHIC ANALYSIS FOR THE FOUR COMMONLY USED ANTIEPILEPTIC DRUGS IN SERUM

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SUMMARY

We describe a simple, sensitive method for the determination of phenobarbital, diphenylhydantoin, carbamazepine and primidone in serum, by use of gas–liquid chromatography with temperature programming. The methylated derivatives of these anticonvulsants were well resolved, as was 5-(*p*-methylphenyl)-5-phenylhydantoin, the internal standard. In this procedure we used an ion-exchange resin for separation of the drug from the serum. The proposed procedure requires only 1.0 ml of serum and can be done in less than 1 h. The lower limit of detection for each of the drugs is 0.5 mg/l. Analytical recoveries of drug from serum were excellent and peak height and concentration were linearly related up to twice the toxic concentration for serum.

INTRODUCTION

Gas–liquid chromatography is widely used for determining antiepileptic drugs[1–3]. Its sensitivity and specificity allow simultaneous measurement of the commonly used antiepileptic drugs. We describe a simple method for simultaneous determination of primidone, diphenylhydantoin, carbamazepine and phenobarbital in 1 ml of plasma or serum in less than 1 h. In this procedure we used the Brinkmann Amberlite™ resin column to extract the antiepileptic drugs from the serum or plasma.

EXPERIMENTAL

Materials

The column used was a Drug Skreen column with Amberlite™ XAD-2 resin (Brinkmann, Westbury, N.Y., U.S.A.). A phosphate buffer (pH 7) was prepared as follows: 60 ml of 0.15 *M* dibasic sodium phosphate solution and 40 ml of 0.15 *M* monobasic potassium phosphate solution were mixed and the pH adjusted to 7.

Drugs used were: diphenylhydantoin (Dilantin; Parke & Davis, Detroit, Mich., U.S.A.), carbamazepine (Tegretol; CIBA Pharmaceutical, Summit, N.J., U.S.A.), phenobarbital (Eli Lilly, Indianapolis, Ind., U.S.A.), primidone (Ayerst

Laboratory, New York, N.Y., U.S.A.) and 5-(*p*-methylphenyl)-5-phenylhydantoin (MPPH; Aldrich, Milwaukee, Wisc., U.S.A.).

Trimethylphenylammonium hydroxide (TMPAH, 0.2 moles/l) was prepared from trimethylphenylammonium iodide (Fisher, Silver Spring, Md., U.S.A.) that had been recrystallized three times from absolute ethanol. To a 250-ml glass-stoppered erlenmeyer flask with a PTFE stirrer, 6.94 g of silver oxide, 10.52 g of trimethylphenylammonium iodide, and 200 ml of absolute methanol were added. Stir (with a magnetic stirrer) for 2.5 h and store at 4°. The supernatant solution is stable for at least a month. Filter appropriate amounts of it through Whatman No. 1 filter paper immediately before use.

Standards

Internal standard. Dissolve 3 μg of MPPH per ml of chloroform, and use 12 ml of this solution for the extraction.

Drug standards. The stock drug standards were prepared in the following concentrations: phenobarbital, 2 g/l; carbamazepine, 0.5 g/l; primidone, 1 g/l; and diphenylhydantoin, 2 g/l. The stock drug standards were added to a drug-free pool of serum to obtain five standards with the concentrations listed in Table I. All four drugs were added to the same serum. These standards were kept frozen in screw-capped, PTFE-coated tubes until use.

TABLE I

CONCENTRATION OF WORKING STANDARDS (mg/l)

Standard	*Phenobarbital*	*Diphenylhydantoin*	*Primidone*	*Carbamazepine*
1	5	2.5	2.5	1
2	10	5	5	2.5
3	20	10	10	5
4	40	20	15	7.5
5	80	40	20	10

Standard curve. Standard curves were prepared for each drug. Each serum standard was extracted and chromatographed in duplicate as if it were a patient sample. The mean value of the relative peak height ratios from the duplicate analyses of each standard were plotted against the concentration of the respective standard (Fig. 1).

Instrumentation

We used a Model 2400 gas chromatograph (Fisher, Pittsburg, Pa., U.S.A.) with dual hydrogen flame ionization detectors and borosilicate glass columns (200 cm × 2 mm I.D.) packed with 3% SP 2250 on 100–120 mesh Supelcoport-type methyl–phenyl (50:50) (Supelco, Bellefonte, Pa., U.S.A.). Before use, the columns were heat conditioned at 310° for 24 h with a carrier gas (nitrogen) flow-rate of 50 ml/min.

The oven temperature was maintained at 190° during periods of inactivity, with a carrier gas flow-rate of 50 ml/min. The injection ports were sealed with 10-mm septums (Supelco). Detectors and injection ports were heated to 310° and 250°,

respectively. The electrometer output was monitored with a Fisher Model 5000 series dual-pen recorder at a chart speed of 25 mm/min. The electrometer was operated at a range of 10^{-11} A/mV and the amplifier output was attenuated at 128.

Chromatographic conditions

The oven temperature was programmed from 190 to 300° at a rate of 15°/min, the program being started immediately after the sample extract was injected. Gas flow-rates were adjusted at 160 ml/min (nitrogen), 30 ml/min (hydrogen), and 300 ml/min (air).

Extraction

The Drug Skreen column was pre-wet with 2 ml of pH 7 phosphate buffer and allowed to drain completely. Serum (1 ml) was added to the column followed by 4 ml of 0.25 *N* HCl. Both were allowed to drain completely. The column was attached to the phase separating filter cartridge. The column and filter assembly were placed to drain into a 15-ml conical centrifuge tube. Chloroform (12 ml) containing 36 μg of internal standard was added to elute the drugs from the resin and collected in the centrifuge tube. All aqueous portions were contained in the filter cartridge and the filtrate (chloroform) was dried with the aid of a warm water bath (40°) and a stream of nitrogen. The dried tubes were closed tightly with a cork until ready to be injected to the gas chromatograph.

Chromatography

The dried residue in the centrifuge tube was reconstituted with 100 μl of TMPAH. This extract (1–2 μl) was chromatographed using temperature programming with the SP 2250 column. Peaks were identified by comparing their relative retention times (relative to the internal reference peak) to known standards. Drug concentrations were calculated from the standard curve.

The precision of the proposed method was checked by using aliquots of spiked serum pools which were kept frozen. The serum pool contained all four drugs. The standard deviation and percentage of recovery are given in Table II.

TABLE II

BETWEEN-DAY ANALYTICAL RECOVERIES ($N = 31$)

Drugs added to serum	*Conc. (mg/l)*	*Mean conc. found (mg/l)*	*S.D. (mg/l)*	*Recovery (%)*
Phenobarbital	20	19.2	1.1	96
Diphenylhydantoin	10	9.2	0.4	92
Primidone	15	14.1	1.0	94
Carbamazepine	10	9.5	0.6	95

RESULTS

Fig. 1 is a standard curve of primidone, diphenylhydantoin, carbamazepine, and phenobarbital prepared by the peak height ratio technique utilizing MPPH as the internal standard. The standards were run in duplicate and the mean value used to

plot the graph. All four standard curves were linear to approximately twice their toxic level. Recovery of the drugs from the serum was measured by adding known amounts of drugs (phenobarbital, diphenylhydantoin, carbamazepine, and primidone) to the same sample and analyzed for 31 consecutive days. The recovery was 92–96% (Table II).

Chromatograms of serum specimens extracted and methylated according to our procedure are presented in Figs. 2, 3 and 4. Fig. 2 is a typical pattern of the serum of a normal individual not receiving anticonvulsants. This was run with the internal standard (I.S.). The four predominant peaks which are due to serum constituents are marked S. Fig. 3 is from a patient who is on all four of the antiepileptic drugs. The concentrations calculated from this serum sample were primidone, 9 mg/l; phenobarbital, 25 mg/l; carbamazepine, 3 mg/l; and diphenylhydantoin, 12 mg/l. Fig. 4 was prepared by adding known drugs to pooled serum. All four drug peaks were well separated and there were no interfering peaks.

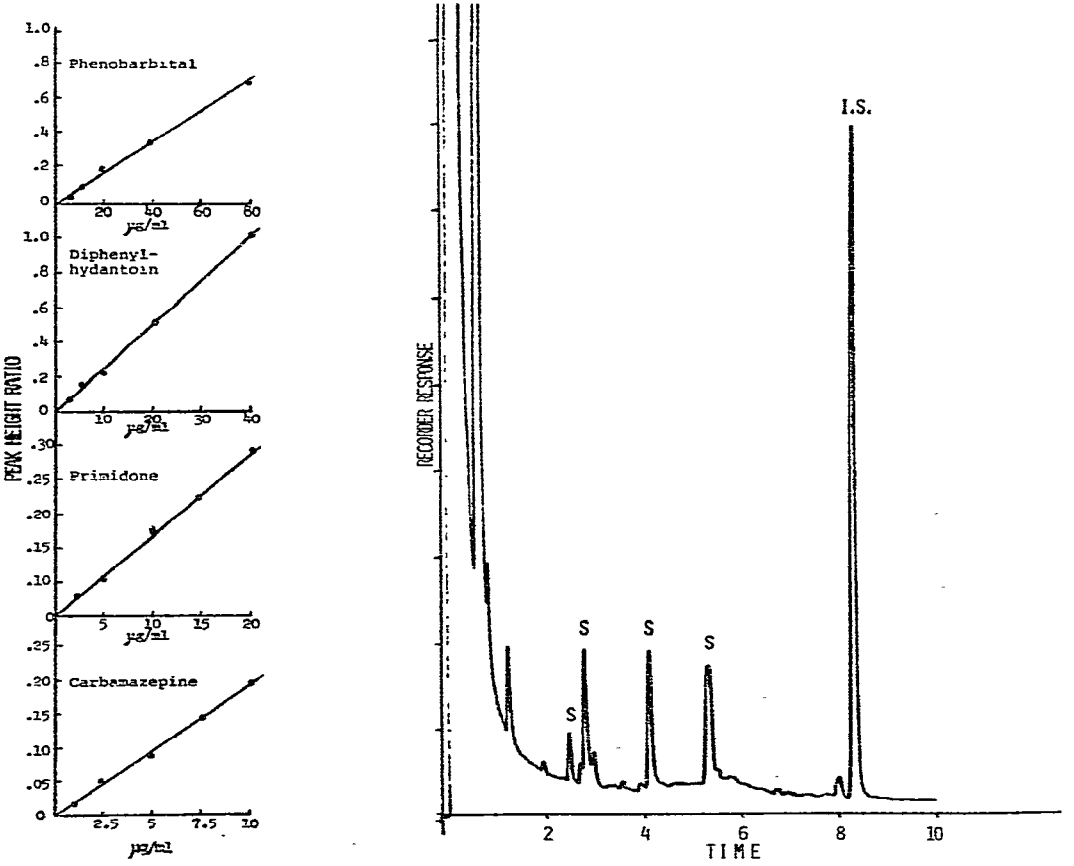

Fig. 1. Standard curves for phenobarbital, diphenylhydantoin, primidone, and carbamazepine.

Fig. 2. Chromatogram obtained by methylation of serum extract from an individual who is not on any antiepileptic drugs. Conditions: see Experimental. S = serum constituents, I.S. = internal standard (MPPH).

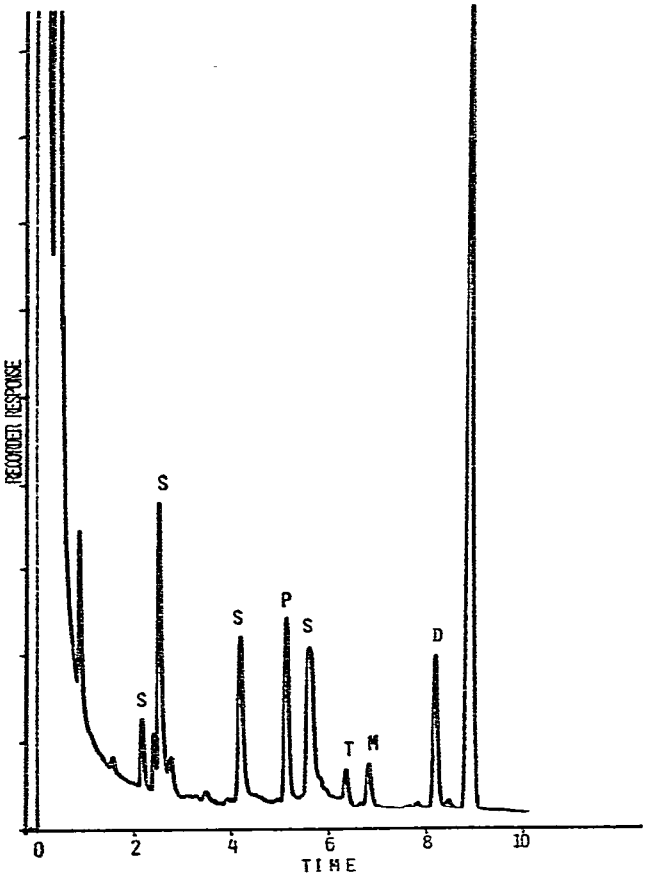

Fig. 3. Chromatogram obtained by methylation of serum extract from a patient who is on four antiepileptic drugs. Conditions: see Experimental. P = phenobarbital, T = carbamazepine, M = primidone and D = diphenylhydantoin, S = serum constituents.

Precision

The precision of the proposed method was evaluated by a between-day (Table II) statistical analysis. The between-day standard deviation varied from 0.4 to 1.1 mg/l. Recoveries of the drugs were good and varied from 92 to 96%.

The usual sample volume used was 1 ml of serum or plasma. In pediatric cases similar results can be obtained by reducing serum, chloroform and TMPAH proportionately. In some instances we used as little as 0.2 ml of serum.

Drug concentrations as low as 0.5 mg/l were easily quantitated by adjusting the sensitivity of the analysis, *e.g.*, injecting a more concentrated extract, increasing the volume of injection or increasing the electrometer output.

DISCUSSION

Rose *et al.*[4] emphasize the fact that the determinations of antiepileptic drugs are not readily available to the majority of physicians who prescribe these medications.

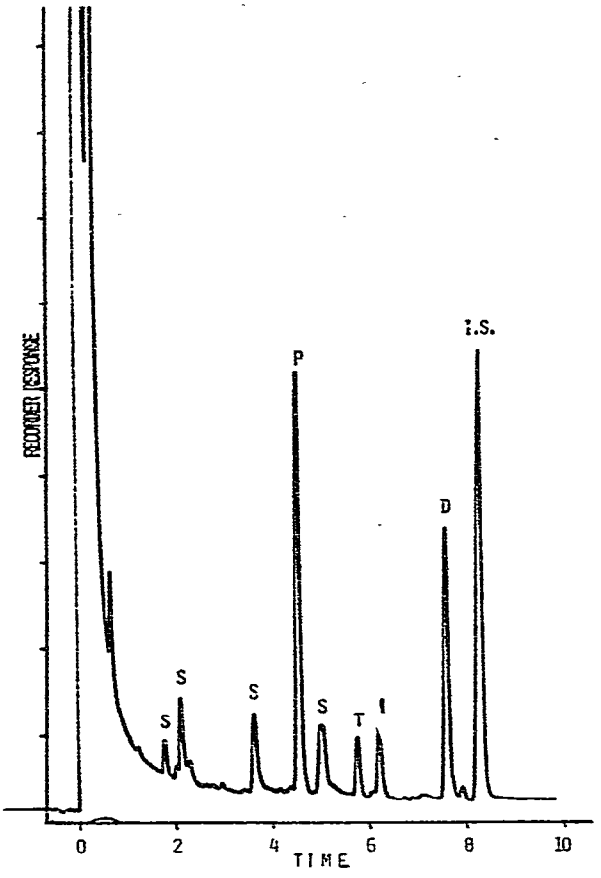

Fig. 4. Chromatogram obtained by methylation of serum extract which was spiked with four of the antiepileptic drugs. Conditions: see Experimental. Identification of peaks as in Fig. 3. I.S. = internal standard (MPPH).

This is primarily because laboratories are reluctant to establish anticonvulsant assays due to the fact that it requires considerable capital investment and commitment of experienced technical personnel. However, it is important to have rapid and accurate blood analyses for better treatment of epilepsy.

The method we offer is simple and well-suited for the clinical laboratory. A single chromatographic system is used for the analysis of all four of the anticonvulsant drug levels. The use of a single internal standard and one temperature program reduces the technical difficulties and standardization procedure. The temperature programming permits well-separated peaks for all of the four drugs. The on-column methylation, use of glass column and SP 2250 gives good peak characteristics and a linear standard curve.

The five standards which contain all the four drugs were prepared on the basis of ref. 5. In this method standard five is approximately twice the toxic level while standard one is less than one-third the therapeutic level.

The most advantageous aspect of the procedure is the capability to determine the most commonly used antiepileptic drugs in a single procedure within 1 h.

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