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IMPROVED METHOD FOR THE SIMULTANEOUS DETERMINATION OF PHENOBARBITAL, PRIMIDONE AND DIPHENYLHYDANTOIN IN PATIENTS' SERUM BY GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

A quantitative gas–liquid chromatographic method for the simultaneous determination of phenobarbital, primidone and diphenylhydantoin in human serum is described. A double extraction is employed to improve the removal of interfering substances. The N,N-dimethyl derivatives of the compounds are prepared by "on-column" methylation with 50% Methelute. Decomposition of phenobarbital to N-methyl-α-phenylbutyramide was negligible if the contact time with Methelute was less than 10 min. The drugs were stable in serum for at least two weeks. This procedure provides a rapid, sensitive, selective and accurate method for the routine determination of serum concentrations of three of the most commonly prescribed anticonvulsant drugs.

INTRODUCTION

The importance of blood level determinations of anticonvulsant drugs is well known[1–6]. Gas–liquid chromatography (GLC) has become the method of choice as it offers the necessary selectivity and sensitivity.

The simultaneous measurement of serum levels of phenobarbital, primidone and diphenylhydantoin in patients has been reported previously[5,7,8]. In our laboratory these methods did not remove sufficient impurities during the extraction procedure to allow adequate serum analysis.

Most procedures use "on-column" methylation for the determination of anticonvulsants[5,7–10]. Several of them employed tetramethylammonium hydroxide[5,9,10]. This methylating agent produced unsatisfactory derivatization in our laboratory. Another author[7] has used trimethylanilinium hydroxide (Methelute). This chemical has been recently shown to decompose phenobarbital[11].

This paper describes a new analytical procedure with improved extraction clean-up and "on-column" derivatization. A study of phenobarbital decomposition by trimethylanilinium hydroxide was carried out. The serum stability of the drugs along with the precision and accuracy of the new method were also investigated.

EXPERIMENTAL

Reagents

All chemicals were of reagent grade. Diphenylhydantoin was donated by Parke, Davis & Co. (Brockville, Canada). Primidone was donated by Ayerst Labs. (Montreal, Canada). Phenobarbitone sodium was purchased from BDH (Toronto, Canada) and 5-(*p*-methylphenyl)-5-phenylhydantoin (MPPH) from Aldrich (Edmonton, Canada). Trimethylanilinium hydroxide 0.2 *M* in methanol ("Methelute", Pierce) was obtained from Chromatographic Specialities (Brockville, Canada).

Solutions

Methylation solution. 50% Methelute was prepared by dilution with methanol.

Phenobarbital, primidone and diphenylhydantoin standard solutions. Methanolic standard solutions were prepared to contain 5, 10, 20, 30, 40, 50 and 60 μg/ml by diluting concentrated stock solutions.

Internal standard solution. A 200 μg/ml solution of MPPH in methanol was prepared.

Apparatus

A Pye Series 104 Model 64 gas chromatograph was used with dual column oven, separately controlled injection port heaters and detector oven, dual hydrogen flame ionization detectors and a linear temperature programmer. The amplifier was connected to a Westronics Model MT 21 recorder.

A 5-ft. × 0.25-in.-O.D. glass column containing 5% OV-17 on 60–80 mesh high-performance Chromosorb W (Chromatographic Specialities) was operated at 160° for 10 min initially, programmed at 8°/min to 215°, and maintained at this temperature for 15 min. The injection port and detector temperatures were 280° and 350°, respectively. The nitrogen carrier gas flow-rate was 60 ml/min, while the hydrogen and air flow-rates were 60 and 400 ml/min, respectively.

Extraction procedure

A flow sheet outlining the extraction procedure is given in Fig. 1.

To a serum sample (2.0 ml) in a 15-ml stoppered glass centrifuge tube is added 0.2 ml of the 200 μg/ml methanolic solution of MPPH and 0.2 ml of 2 *N* HCl to make the solution acidic. After thorough mixing, the sample is extracted with two 5.0-ml portions of chloroform by shaking for 5 min on a Cenco-Weinzer laboratory shaker, followed by centrifugation at 350 *g* for 5 min. The lower organic layers are removed with a Pasteur pipette and combined in a 15-ml test tube. The pooled chloroform extract is concentrated to approximately 5 ml by directing a stream of dry nitrogen over the chloroform at 70°. The compounds are then extracted into an aqueous medium by vortexing for 1 min with 2.0 ml of 0.5 *N* NaOH followed by centrifugation at 350 *g* for 5 min. The upper aqueous phase is removed and saved. The extraction is then repeated with an additional 2.0 ml of 0.5 *N* NaOH and combined with the initial basic extract. The aqueous phase is acidified with 1.5 ml of 2 *N* HCl and extracted with 4.0 ml of diethyl ether by vortexing for 1 min, followed by centrifugation at 350 *g* for 5 min. The upper ethereal layer is transferred to a 15-ml centrifuge tube. The extraction is then repeated with two additional 4.0-ml portions of diethyl ether and the extracts are com-

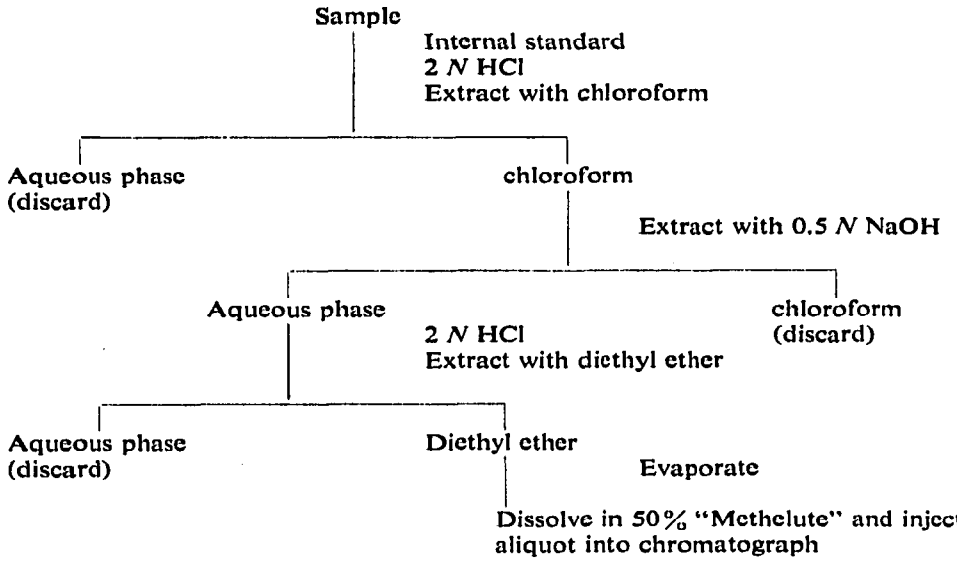

Fig. 1. Flow sheet outlining the extraction procedure of phenobarbital, primidone and diphenylhydantoin from serum.

bined. The extract is concentrated by evaporating the diethyl ether to dryness by directing a stream of nitrogen over the diethyl ether. The sample extract is concentrated in the lower tip of the tube by rinsing the sides of the tube with 0.2 ml of methanol followed by evaporation to dryness. The sample is dissolved in 20 μl of 50% Methelute and 1 μl of the solution is injected into the gas chromatograph within 10 min.

Standard curves

Standard calibration curves were prepared by the addition of phenobarbital, primidone and diphenylhydantoin in the range of 5–60 μg/ml and 20 μg/ml of MPPH to blank serum. The peak height ratios of the compounds to the internal standard were plotted against concentration to give the standard curves. Each calibration curve was constructed from duplicate determinations of seven different points. The curves were linear in the range examined, which allowed the use of the peak height ratios for the determination of unknown samples.

RESULTS AND DISCUSSION

A typical chromatogram of a serum sample containing phenobarbital, primidone, diphenylhydantoin and the internal standard is shown in Fig. 2.

Extraction procedure

MacGee[9] used MPPH, an analogue of diphenylhydantoin, as the internal standard in the extraction solvent. In this procedure MPPH is added to the serum before extraction. This was found to decrease the necessity for accurate aliquot measurements during extraction and chromatography. The standard curves were linear in the range of 5–60 μg/ml, indicating that the drugs and internal standard appeared to

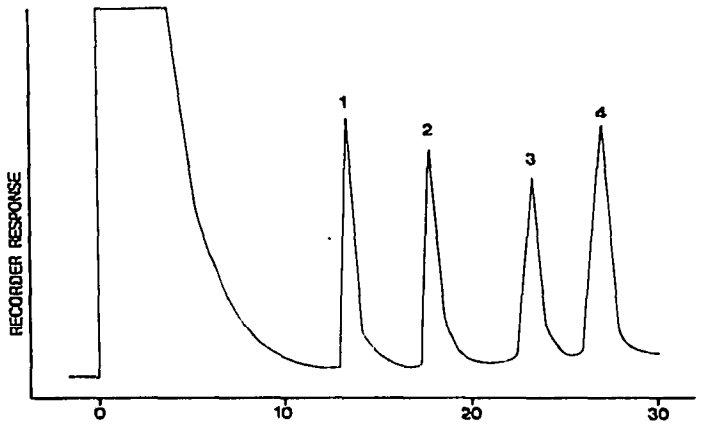

Fig. 2. Gas chromatogram of the methylated derivatives of phenobarbital (1), primidone (2), diphenylhydantoin (3) and the internal standard 5-(*p*-methylphenyl)-5-phenylhydantoin (4).

be extracted and derivatized with the same efficiency over a large range of serum levels.

The measurement of the compounds was attempted after extraction with chloroform according to the available methods[5,7,8,10,12,13]. The chromatogram contained unknown peaks with similar retention times to phenobarbital and primidone. The accurate measurement of the peak heights was not possible due to the interference peaks. The incorporation of a back extraction, followed by acidification and re-extraction into diethyl ether removed the interfering peaks and allowed greater accuracy of measurement of the peak heights.

Decomposition of phenobarbital

Osiewicz *et al.*[11] reported that the methylating agent, trimethylanilinium hydroxide, produced variable results with phenobarbital due to hydrolytic breakdown of the phenobarbital by the derivatizing agent. For this reason the effect of

TABLE I

EFFECT OF TIME OF CONTACT ON THE PRODUCT RATIO OF PHENOBARBITAL TO INTERNAL STANDARD

Contact time	*Product ratio*
1 min	1.91
2 min	1.99
3 min	1.98
4 min	1.94
5 min	2.00
10 min	1.90
	Av. 1.95 ± 0.04
	C.V. 1.8%
30 min	1.79
1 h	1.21
24 h	1.27

TABLE II

EFFECT OF CONCENTRATION OF METHELUTE ON THE PRODUCT RATIO OF PHENOBARBITAL TO INTERNAL STANDARD

% Methelute	*Methelute concentration (M)*	*Product ratio*
100	0.2	1.42
50	0.1	1.99
25	0.05	1.93
10	0.02	1.54
5	0.01	0.96

trimethylanilinium hydroxide on the recovery of phenobarbital against exposure time was studied. Standard solutions containing 20 μg/ml of phenobarbital and MPPH were analyzed at various time intervals, as indicated in Table I. If the samples were chromatographed within 10 min after the addition of 50% Methelute, the ratio was not significantly altered. However, if the contact time of the sample with 50% Methelute was more than 10 min, the product ratio decreased and was variable.

Concentration of derivatizing solution

The phenobarbital derivative was eluted before the solvent front had returned to the original baseline in the chromatogram when 100% Methelute was used (Table II). The peak height ratios varied and were lower for similar concentrations. 50% Methelute decreased the size of the solvent peak and ensured good separation of the phenobarbital derivative and the solvent front. The product ratios were reproducible when 25–50% Methelute was used. Lower concentrations of Methelute gave varying results.

Precision and accuracy

A mixture of the drugs and serum containing 20 μg/ml of phenobarbital, 20

TABLE III

EFFECT OF STORAGE ON THE CONCENTRATION OF PHENOBARBITAL, PRIMIDONE AND DIPHENYLHYDANTOIN IN A SERUM SAMPLE

Time (days)	*Concentration (μg/ml)*		
	Phenobarbital	*Primidone*	*Diphenylhydantoin*
1	19.8	10.6	10.1
2	20.3	10.3	10.3
3	19.5	9.7	9.7
4	19.9	9.9	10.0
5	20.4	10.1	9.6
8	20.1	10.4	10.2
9	20.7	9.8	10.3
10	19.5	10.3	9.9
11	19.9	10.1	10.2
12	20.3	9.7	10.4

μg/ml of primidone and 10 μg/ml of diphenylhydantoin was analyzed ten times to determine the reproducibility of the method. The phenobarbital level averaged 19.87 $\pm$ 2.02 (S.D.), the levels of primidone and diphenylhydantoin, 20.27 $\pm$ 3.00 and 9.58 $\pm$ 0.85, respectively.

Stability

The stability of the compounds in serum was investigated by spiking blank serum with 20, 10 and 10 μg/ml of phenobarbital, primidone and diphenylhydantoin respectively. An aliquot of the refrigerated sample was routinely analyzed for twelve days. The results are reported in Table III and show that storage over that period did not produce loss of drugs.

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