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SIMULTANEOUS DETERMINATION OF ANTICONVULSANT DRUGS BY GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

A quantitative gas–liquid chromatographic method for the determination of blood levels of ethosuximide, phenobarbitone, primidone and diphenylhydantoin is described. All four compounds are determined using 1 ml of serum. A simple, direct extraction technique is employed. Ethosuximide is analysed without derivatization. Subsequent flash alkylation with trimethylanilinium hydroxide allows the simultaneous determination of phenobarbitone, primidone, and diphenylhydantoin.

INTRODUCTION

In recent years the monitoring of blood levels of anticonvulsant drugs in patients undergoing chronic therapy has become routine practice in many clinical laboratories. During this time, gas–liquid chromatographic methods of high sensitivity and specificity have been devised and this approach is the current method of choice. Several methods have been reported, most of which determine primidone (5-ethyl-dihydro-5-phenyl-4,6(1H,5H)-pyrimidinedione), phenobarbitone (phenylethylmalonyl-urea), and diphenylhydantoin (5,5-diphenyl-2,4-imidazolidinedione) simultaneously[1–5].

Optimally these routine assays should involve the minimum number of operations on a small volume of plasma or serum whilst producing accurate results and maintaining good long-term precision. These constraints preclude double extraction and back extraction techniques which are included to remove extraneous peaks. By definition, the resolving power of an appropriate chromatographic system should make such time-consuming operations redundant. The maximum sample size should not exceed 1 ml of serum or plasma to include analysis of pediatric samples. Finally, when required, a simple, reliable procedure must be included to ensure the production of volatile derivatives and thereby symmetrical peaks on commonly available liquid phases.

We describe a method for the simultaneous determination of phenobarbitone, primidone, and diphenylhydantoin that meets the requirements outlined above. The

determination of ethosuximide (2-ethyl-2-methylsuccinimide), which is less frequently prescribed for anticonvulsant therapy, is also incorporated into the method, albeit not simultaneously with the other compounds.

EXPERIMENTAL

Apparatus

The gas chromatograph is a Hewlett-Packard Model 5780 equipped with dual flame ionization detectors and dual electrometers. The output from each electrometer is connected to separate channels on a System IVB integrator (Autolab) and in series to a 1-mV recorder (Houston Omniscribe).

Two coiled glass columns (6 ft. × $^1/_8$ in. O.D.) are used, one packed with 3% OV-17 on Gas-Chrom Q (100–120 mesh) and the other with 4% OV-225 on Gas-Chrom Q (100–120 mesh) (Chromatographic Specialties), conditioned for 24 h at 275° with a nitrogen flow-rate of 25 ml/min.

Sample injection is "on column"; the detector temperature is 300°. The carrier gas flow-rate is 22 ml/min on each column; auxiliary nitrogen is added at the detectors to give a total nitrogen flow-rate of 51 ml/min. The hydrogen and air flow-rates are 39 and 240 ml/min, respectively.

Ethosuximide is analyzed isothermally (150°) on the OV-225 column. The methyl derivatives of phenobarbitone, primidone, and diphenylhydantoin are analysed on the OV-17 column operated at 205° for 8 min and then linearly programmed at 4°/min to 220° and maintained at this temperature for 10 min.

Reagents

All chemicals were reagent grade. Diphenylhydantoin and ethosuximide were donated by Parke, Davis and Co. (Brockville, Canada), hexobarbitone was from May & Baker (Toronto, Canada), phenobarbitone was purchased from BDH (Toronto, Canada), and primidone was from Ayerst Lab. (Montreal, Canada). α,α-Dimethyl-β-methylsuccinimide and 5-(*p*-methylphenyl)-5-phenylhydantoin were purchased from Aldrich (Milwaukee, Wisc., U.S.A.) and trimethylanilinium hydroxide (TMAH) at a concentration of 0.2 *M* in methanol ("Methelute"; Pierce, Rockford, Ill., U.S.A.) was from Chromatographic Specialties (Brockville, Canada).

Plasma standards

Ethosuximide (50 mg), phenobarbitone (40 mg), primidone (25 mg), and diphenylhydantoin (sodium salt, 27 mg) were dissolved in 10 ml of anhydrous methanol. 2 ml of this stock solution were made up to 100 ml with outdated blood bank plasma. This standard (A, Table I) was used to prepare serial dilutions in plasma (B, C, D, Table I). Aliquots (*ca.* 2 ml) of these preparations were kept at −20° prior to use in an analytical run. Storage at this temperature was uneventful. New standards were prepared only when a prior batch was exhausted. The incoming batch was routinely assayed against the outgoing batch. The average life-time of a batch depended upon the number of analytical runs and the batch size, but has not exceeded 1 month.

Internal standards

α,α-Dimethyl-β-methylsuccinimide (10 mg), hexobarbitone (5 mg), and 5-(*p*-methylphenyl)-5-phenylhydantoin (6 mg) were dissolved in chloroform (1 l).

TABLE I

CONCENTRATION (mg/l) OF DRUGS IN PLASMA STANDARDS

The concentration range of these standards embraces the reported therapeutic range (ref. 6) for each drug, *i.e.*, phenobarbitone (20–50 mg/l), primidone (4–8 mg/l), diphenylhydantoin (10–20 mg/l), ethosuximide (40–110 mg/l).

Drug	*Standard*			
	A	*B*	*C*	*D*
Phenobarbitone	80	40	20	10
Primidone	36*	18*	9*	4.5*
Diphenylhydantoin	50	25	12.5	6.25
Ethosuximide	100	50	25	12.5

* The purity of our primidone preparation as checked by UV spectroscopy, $E^{1\%}_{1cm}$ = 6.0 at 258 nm, was found to be 72%.

Of this preparation 200 ml were made up to 1 l with chloroform and this solution served as the extraction solvent. α,α-Dimethyl-β-methylsuccinimide was the internal standard for ethosuximide, hexobarbitone that for phenobarbitone, whilst the hydantoin derivative served for primidone and diphenylhydantoin.

Derivatizing reagent

TMAH, 0.2 *M* in methanol was used to prepare volatile methylated derivatives of phenobarbitone, primidone, and diphenylhydantoin.

Extraction procedure

Serum or plasma (1 ml) is added to a 50-ml glass tube fitted with a PTFE-lined screw cap, acidified with 0.5 ml of 0.5 *N* HCl and extracted with 10 ml of chloroform containing the internal standards. The extraction for 10 min (Buchler Omni-shaker) is followed by centrifugation at 500 *g* for 5 min. The aqueous phase is removed by aspiration and the chloroform decanted into a disposable glass tube (15 ml). The solution is taken to dryness by warming under a stream of dry nitrogen, and the residue dissolved in a few drops of methanol. If ethosuximide is part of the analysis, 2 μl of this solution are injected onto the OV-225 column and chromatographed isothermally at 150°. The remaining material is derivatized by adding 100 μl of 0.2 *M* TMAH in methanol and flash heating at 100° for 10 min. The residue is redissolved in a small volume of methanol (*ca.* 50 μl) and 1 μl is injected onto the OV-17 column and chromatographed as described.

The above procedure is followed for patient and standard samples. A standard curve is constructed daily by plotting the peak area ratios of the drugs to their internal standards against the drug concentrations in each standard (see Table I). The level of drugs in an unknown sample is derived from this curve.

RESULTS

Typical chromatograms of plasma samples containing ethosuximide, phenobarbitone, primidone, and diphenylhydantoin are shown in Figs. 1 and 2. Fig. 3 shows typical standard curves for these compounds. Data on precision and accuracy

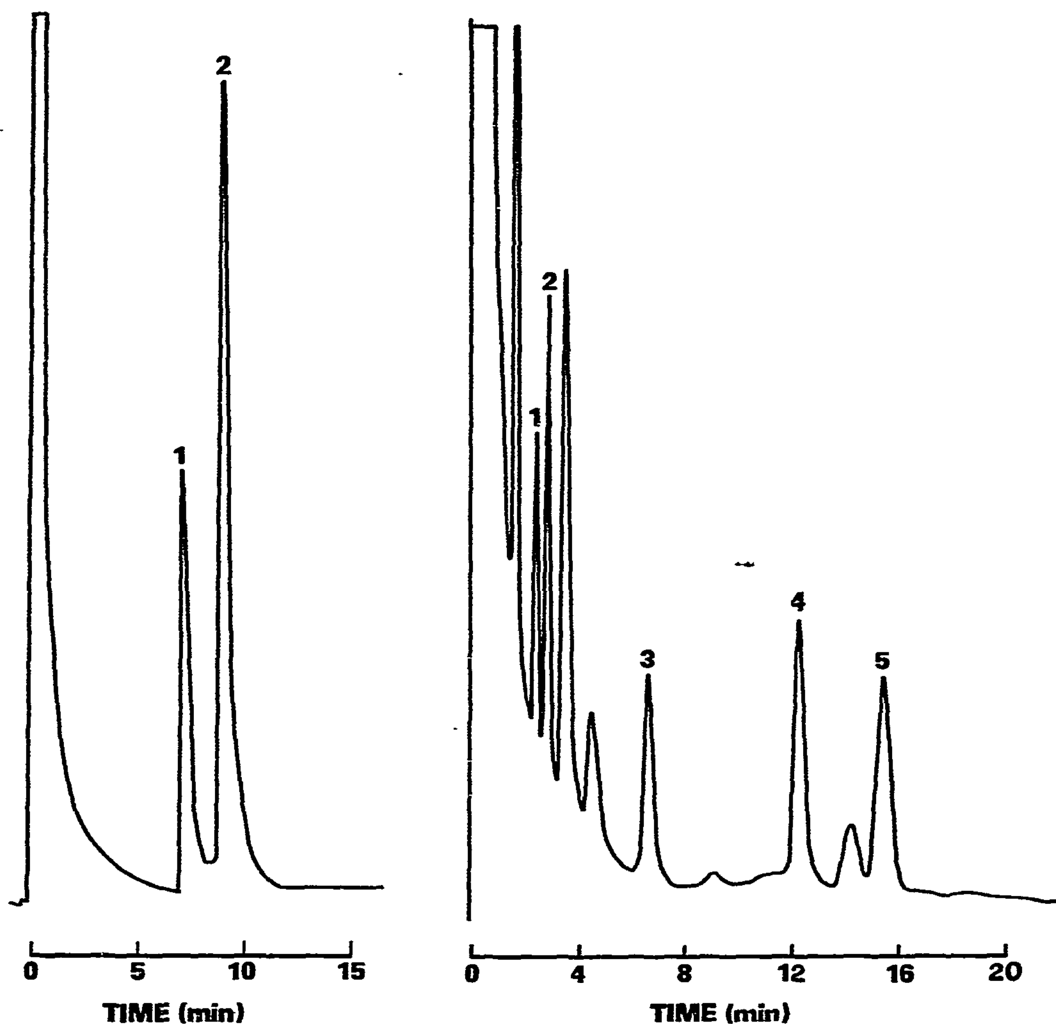

Fig. 1. Gas chromatogram of underivatized α,α-dimethyl-β-methylsuccinimide (1) and ethosuximide (2).

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

have been collected over a three-month period by including a pool sample in each run. These results are outlined in Table II.

DISCUSSION

There are several features of this method worthy of attention. First, the one-step extraction procedure requires little time. In the subsequent chromatography the compounds of interest are sufficiently separated from endogenous materials and the data reported above show that accurate and precise quantitation is easily achieved.

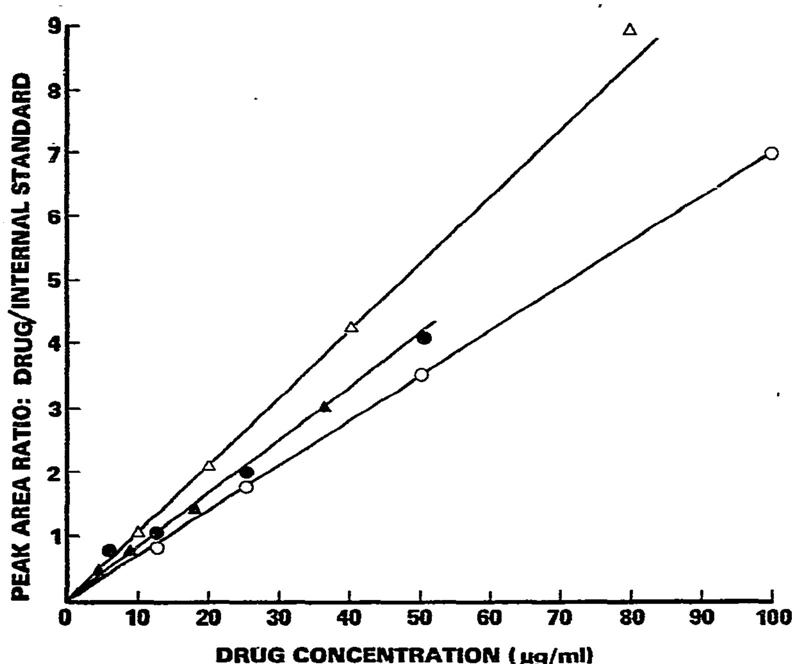

Fig. 3. Standard curves of drug concentration (μg/ml) against peak area ratio of drug to internal standard, △, Phenobarbitone; ▲ and ●, primidone and diphenylhydantoin; ○, ethosuximide.

TABLE II

ACCURACY AND PRECISION DATA OVER A THREE-MONTH PERIOD

Drug	*Concentration weighed (mg/l)*	*Number of samples analysed*	*Concentration found (mean ± S.D.)*	*Coefficient of variation (%)*
Phenobarbitone	40	55	42.64 ± 3.42	7.9
Diphenylhydantoin	25	50	25.80 ± 1.94	7.5
Primidone	18	52	18.38 ± 1.63	8.9
Ethosuximide	50	34	51.42 ± 4.18	8.2

In our experience there has been no instance of miscalculation from interfering drugs in sera from patients on controlled anticonvulsant therapy. An exhaustive investigation of those materials which might interfere and which might be removed by back extraction has not been undertaken, however.

The inclusion of the internal standards in the extraction chloroform minimizes the number of operations in the procedure. Separate addition of internal standards as small volumes of aqueous or methanolic solutions leads to extra pipetting errors and outright blunders if missed altogether from any sample. Addition of the chloroform (10 ml) plus internal standards from an automatic dispenser (precision ± 0.5%) minimizes pipetting errors and precludes blunders.

As yet, there seems to be no concensus of opinion regarding the merits of derivatization for the assay of phenobarbitone by gas chromatography and an earlier meth-

od, reported from this laboratory[7] did not include derivatization. However, our subsequent experience demonstrates that derivatization as described below allows simultaneous chromatography of phenobarbitone, diphenylhydantoin and primidone, thereby improving the efficiency of the overall method, and furthermore, in our hands, improving the long-term quality control of the phenobarbitone determinations.

The method of derivative preparation is also important for the accurate analysis of barbiturates. Alkylation of functional groups is a common procedure to produce volatile derivatives for gas chromatography[8]. Recently, TMAH has become the reagent of choice in many laboratories[8]. However, since TMAH is strongly alkaline and since phenobarbitone and its methylated derivative are both unstable at alkaline pH, two products, N,N-dimethylphenobarbitone and N-methyl-α-phenylbutyrimide (early phenobarbitone), are produced when alkylation is over a prolonged time (10 min) at room temperature[5]. Clearly, for accurate and precise quantitation, this effect should be avoided. Rapid injection of the mixture into the chromatograph destroys the TMAH by Hofman elimination and simultaneously induces "on column" methylation of the phenobarbitone to the N,N-dimethyl derivative. The remaining sample, however, remains in the alkaline environment and any further injection, if required, produces multiple peaks and cannot be compared with the first. Flash alkylation "off column"[9] is a rapid, simple method for producing complete methylation of barbiturates (including the hexobarbitone internal standard) and hydantoins to single derivatives which are then stable in the subsequent neutral environment for at least 24 h at room temperature.

The routine sample size is 1 ml. However, since the chromatograph is normally operated at 80 times below its upper limit of sensitivity, the method can easily be applied to smaller samples. Currently, 0.5-ml samples are often analysed without loss in accuracy or precision even without changing the instrument parameters.

Finally, the data presented in Table II represent information generated over a three-month period from analyses performed by a large (approximately 30 people) rotating staff, and as such reflects the true operating quality control of this method.

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