SIMULTANEOUS QUANTITATIVE GAS-CHROMATOGRAPHIC ANALYSIS OF ETHOSUXIMIDE, PHENOBARBITONE, PRIMIDONE AND DIPHENYLHYDANTOIN

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1 Therapeutic serum concentrations of ethosuximide, phenobarbitone, primidone, and diphenylhydantoin were assayed from 1 ml of human serum. The extraction procedure was common to all four drugs and three internal standards.

2 Subsequent isothermal gas chromatographic analysis of serum extracts produced well resolved peaks for the underivatized quantitation of ethosuximide and phenobarbitone. Primidone and diphenylhydantoin were determined as methylated derivatives.

3 Mean coefficients of variation for the assay of each drug were less than 7% on a newly packed and conditioned column and less than 10% after the technique had been in continuous use for 3 months.

4 The advantage of quantitation relative to peak area ratios rather than peak height ratios was minimal for the determination of ethosuximide, primidone and diphenylhydantoin but appeared significant for the assay of phenobarbitone.

Introduction

The high degree of specificity and sensitivity of gas liquid chromatography (g.l.c.) has, for several years, favoured this method of quantitative drug analysis. In addition, the results of an international quality control scheme suggested to Richens (1975) that g.l.c. procedures were the most accurate for the routine determination of serum anticonvulsant concentrations. At the same time, however, these studies suggested there was considerable room for improvement in the methods of routine g.l.c. analysis available.

Many g.l.c. methods have been described for the quantitation of anticonvulsant drugs either singly or in various combinations (Pippenger & Gillen, 1969; Kupferberg, 1970; MacGee, 1970; Papadopoulos, Baylis, Fry & Marks, 1973). In addition to the advantages of a common extraction procedure a multi-drug analysis, for which a minimal volume of blood is required, has particular application in paediatrics. Most combined methods have estimated various combinations of diphenylhydantoin, phenobarbitone and primidone and occasionally other

anticonvulsant drugs (e.g. pheneturide and carbamazepine) after extraction from plasma or serum into a suitable organic solvent. Recently, Van der Kleijn, Collste, Norlander, Agurell & Sjoqvist (1973) described a method for the quantitative determination of ethosuximide. By means of linear temperature programming, trimethadione, phensuximide, phenobarbitone, primidone and nitrazepam were also identified. The more recent techniques usually include a back extraction into alkali in order to remove compounds other than anticonvulsant drugs which produce interfering peaks on the final chromatogram. After extraction, drugs have been chromatographed either with (Kupferberg, 1970; Baylis, Fry & Marks, 1970; Miyumoto, Seino, Ikeda & Yamagami, 1973) or without (Pippenger & Gillen, 1969; Toseland, Grove & Berry, 1972; Papadopoulos *et al.*, 1970) the prior formation of less polar derivatives.

This paper describes an extension of the method of Latham & MacPherson (1974) for the quantitation of ethosuximide to incorporate, in

addition, a reliable quantitative assay for phenobarbitone, primidone and diphenylhydantoin from 1 ml of human serum.

Methods

Extraction procedure

Serum (1 ml) containing one or more of the anticonvulsant drugs ethosuximide, phenobarbitone, primidone and diphenylhydantoin was added to the appropriate internal standards (100 μ l of each) plus 0.5 N HCl (0.5 ml) contained in a silylated glass centrifuge tube fitted with a teflon-lined screw cap. The internal standards, prepared in methanol, were α - α -dimethyl- β -methyl succinimide (DMMS, 400 μ g/ml for ethosuximide), hexobarbitone (200 μ g/ml for phenobarbitone) and 5-(*p*-methylphenyl)-5-phenylhydantoin (MPPH, 250 μ g/ml for primidone and diphenylhydantoin). If a certain drug was known to be absent from a particular serum specimen, the requisite internal standard was omitted. Chloroform (10 ml) was added and the mixture shaken gently for 3 min and then centrifuged at 2,000 rev/min for 10 minutes. The organic layer was subsequently transferred by means of a silylated pasteur pipette, to a second silylated centrifuge tube and evaporated to a volume of approximately 100 μ l over a warm sand tray under a stream of nitrogen. Excess methanol (approximately 0.5 ml) was then added and the contents of the tube well mixed before further reducing the volume to about 30 μ l.

Chromatography

Assays were performed on a Hewlett Packard (Model 402) gas chromatograph fitted with dual flame ionization detectors. The 6 ft silylated glass U columns (1/4 in o.d.) were packed with 3% OV 17 on Gas Chrom Q (Applied Science Laboratories). For all parts of the assay the hydrogen and air flow rates were constant at 37 and 300 ml/min respectively. A nitrogen (carrier gas) flow rate of 30 ml/min was used for the ethosuximide assay and 60 ml/min for the determination of phenobarbitone, primidone and diphenylhydantoin.

Ethosuximide and DMMS were chromatographed at an oven temperature of 130°C and injection port temperature of 150°C after injection of a 1-2 μ l aliquot of the extract onto the column (Figure 1a).

The oven temperature was increased to 210°C and injection port to 250°C. A second 1-2 μ l aliquot of the extract was subsequently injected

Figure 1 Chromatograms for the quantitative determination of (a) ethosuximide (retention time 2.33 min) against DMMS (retention time 1.96 min). (b) phenobarbitone (retention time 3.28 min) against hexobarbitone (retention time 1.78 min). (c) primidone (retention time 3.29 min) and diphenylhydantoin (retention time 6.56 min) against MPPH (retention time 9.24 minutes). The arrows indicate the points of injection.

onto the column in order to quantitate phenobarbitone (Figure 1b).

Trimethylanilinium hydroxide in methanol (10 μ l of a 0.1M solution) was added to the remainder of the original extract which was subsequently reduced to a final volume of approximately 10 μ l under nitrogen. Maintaining the oven temperature at 210°C and injection port at 250°C (as for the chromatography of phenobarbitone) an injected aliquot of 1-2 μ l produced well resolved peaks for the methylated derivatives of primidone, diphenylhydantoin and MPPH (Figure 1c).

Routine analysis

The limiting factor in the number of assays that could be performed during a working day was the time required to chromatograph the extracts. Successive extracts containing all four drugs were chromatographed at intervals of 5 min for ethosuximide, 30 min for phenobarbitone and 11 min for the combined primidone and diphenylhydantoin estimations. It was necessary to allow 30 min between phenobarbitone injections so that extraneous material did not

interfere with subsequent drug peaks. Approximately 2 h were required to extract a batch of eight serum specimens or standards containing all four drugs. Allowing for restabilization of the gas chromatograph after the estimation of ethosuximide, 6.5 h were necessary for the chromatography of the thirty-two unknown drug concentrations when each extract contained all four drugs.

Reproducibility studies

Variation attributable to different stages of the method was assessed for each drug as a function of the reproducibility of the complete method, chromatography and extraction procedure.

(a) *Complete method* A standard solution of the four anticonvulsant drugs in serum was extracted eight times, each extract subsequently being chromatographed for each of the drugs using the specific chromatographic conditions required for the assay.

(b) *Chromatography* A methanolic solution containing the four anticonvulsant drugs and three internal standards, in suitable relative concentrations, was prepared. Aliquots (1-2 μ l) of this solution were repeatedly injected onto the column which was previously stabilized at the

predetermined conditions for the assay of ethosuximide and phenobarbitone. Excess trimethylaluminium hydroxide was added to the remainder of the solution and aliquots were chromatographed for derivatized primidone and diphenylhydantoin.

Preparation of standards

A stock solution containing all four drugs was prepared by adding 0.5 ml of a solution of the drugs in methanol to 100 ml pooled human serum. The serum standard thus prepared was serially diluted with human serum to produce a range of suitable working standards each of which was assayed, against the appropriate internal standard, in duplicate.

Results

Reproducibility studies

The mean coefficient of variation of each drug assay (calculated from peak height ratios) was determined for the extraction procedure, chromatography on a newly packed and conditioned column and complete method (Table 1). After regular use of the column for three months the study was repeated (Table 2). In

Table 1 Variation in the extraction and chromatography of ethosuximide, phenobarbitone, primidone and diphenylhydantoin. Figures are based on peak height ratios calculated from reproducibility studies using a newly packed and conditioned column

Drug	Mean coefficient of variation (%)		
	Complete method	Chromatography	Extraction
Ethosuximide	3.3	3.6	-0.3
Phenobarbitone	4.3	3.7	0.6
Primidone	6.5	4.4	2.1
Diphenylhydantoin	4.3	3.5	0.8

Table 2 Variation in extraction and chromatography (on a column which had been in use routinely for 3 months) of ethosuximide, phenobarbitone, primidone and diphenylhydantoin. Figures are based on peak area ratios calculated by a Hewlett Packard 3380A series integrator and peak height ratios determined from the same chromatograms

Drug	Mean coefficient of variation (%)					
	Complete method		Chromatography		Extraction	
	Area	Height	Area	Height	Area	Height
Ethosuximide	2.2	3.8	0.4	4.6	1.8	-0.8
Phenobarbitone	9.3	17.7	5.7	15.5	3.6	2.2
Primidone	5.2	3.6	1.6	1.6	3.6	2.0
Diphenylhydantoin	4.1	2.8	1.1	1.4	3.0	1.4

Figure 2 Standard curve for the g.l.c. estimation of ethosuximide based on peak height ratio measurements.

Figure 4 Standard curves for the g.l.c. estimation of primidone (○) and diphenylhydantoin (●) based on peak height ratio measurements.

Figure 3 Standard curve for the g.l.c. estimation of phenobarbitone based on peak height ratio measurement (●) using a newly packed and conditioned column and peak area ratio measurements (○) on a column which had been used routinely for 3 months.

addition to peak height ratios, peak areas were also determined on this occasion using a Hewlett Packard 3380A series integrator.

Standard curves

Graphs of serum drug concentrations were initially determined relative to peak height ratios

(drug/internal standard). A linear relationship was shown for all four drugs (Figures 2, 3 and 4). Because of the decreasing reproducibility of the phenobarbitone assay with time when based on peak height ratios, the standard curve for this drug was repeated and the serum phenobarbitone concentration related to integrated peak area ratio measurements (Figure 3).

Specificity

Injected aliquots of methanolic solutions of pheneturide, sulthiame, chlordiazepoxide and diazepam caused no interference under any of the conditions described for the chromatography. Carbamazepine, however, manifested a peak with a retention time identical to that of the hexobarbitone internal standard.

Discussion

The method described for the quantitative analysis of ethosuximide, phenobarbitone, primidone and diphenylhydantoin incorporated many of the advantages of previously described procedures. Most important of these was the use of 1 ml of serum from which all four drugs were assayed. Although other techniques have estimated one or more anticonvulsants *via* a single extraction procedure the drugs assayed have not necessarily been the most commonly used in the treatment of epilepsy. Surprisingly, no previous method has adopted derivatization of the final fraction of the extract in order to facilitate the determination of

primidone and diphenylhydantoin after the assay of underivatized ethosuximide and phenobarbitone. Quantitation from a single extract of 1 ml of serum minimized the time taken for the extraction procedure and was found to be particularly applicable to the paediatric situation. Furthermore, the simplicity of the single extraction technique was thought to be important in obviating human error when performing assays routinely. A back extraction was not required to eliminate interfering peaks on the final chromatogram. Although the incorporation of this step might have reduced the chromatographic assay time of phenobarbitone, it would have complicated the simultaneous extraction of primidone.

Phenobarbitone was the most difficult drug to assay and produced the greatest chromatographic and extraction sources of error even though every precaution was taken to eliminate losses due to binding of the drug to glassware, which was treated with a 5% solution of dimethyldichlorosilane in toluene at weekly intervals. The increasing chromatographic variation in peak height ratios relative to peak area ratios with ageing of the column for the phenobarbitone assay cannot be adequately explained. This effect, however, emphasizes the importance of measuring peak area rather than peak height ratios for the

assay of phenobarbitone and stresses the importance of frequent quality control in assay procedures. The interference of carbamazepine with hexobarbitone on the final chromatogram (i.e. identical retention times) was unimportant in view of the relatively infrequent use of this drug in the treatment of epilepsy. It could be argued, however, that this is one limitation of the method.

Many of the practical problems incurred in the gas-chromatographic analysis of anticonvulsant drugs were reviewed by Van Meter (1973). Furthermore, Meijer (1973) implied that the difficulties encountered in the assay of anticonvulsant drugs were considerable and controversial and suggested that an accuracy of $\pm 10\%$ in the determination of serum concentrations should suffice as a guide to therapy. The present method, being within this specification, could therefore be valuable when monitoring serum ethosuximide, phenobarbitone, primidone and diphenylhydantoin concentrations in a routine clinical chemistry laboratory.

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