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GAS CHROMATOGRAPHIC ANALYSIS OF PHENYLETHYLMALONAMIDE IN HUMAN PLASMA

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SUMMARY

A method is described for the analysis of phenylethylmalonamide in human plasma. Analysis of plasma requires only 200 μl of sample which is extracted with dichloroethane. After filtration and evaporation of the solvent the residue is reconstituted in 50 μl of chloroform and 5 μl are injected onto the gas chromatograph. The column used is a mixture of CDMS/WG11 coated on Chromosorb W HP 100–120 mesh. The method is suitable for use in single-dose pharmacokinetic studies.

INTRODUCTION

Primidone was introduced as an anticonvulsant in 1952 and is a valuable drug for the treatment of generalised and psychomotor seizures [1]. In humans it is metabolised to produce phenobarbitone and phenylethylmalonamide (PEMA) (Fig. 1), both of which accumulate in blood since each is cleared from plasma at a lower rate than the parent compound.

Primidone is administered orally and is usually efficiently absorbed. In animal studies approximately 50% of the dose is excreted as PEMA in the urine and 20% is eliminated unchanged [2]. Of the remainder some is eliminated as phenobarbitone, but the majority as hydroxyphenobarbitone which is excreted predominantly as its *o*-glucuronide conjugate.

Phenobarbitone possesses anticonvulsant properties and its pharmacological activity is related to the concentration of the drug in plasma. However, large inter-individual variations exist in this plasma level–effect relationship. Little is known about the significance of either the plasma concentration or the pharmacological activity of PEMA, although there are conflicting reports about its

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TABLE I

SUMMARY OF SOME GC PROCEDURES FOR THE MEASUREMENT OF PEMA

Sample volume (ml)	Internal standard	Derivatisation	Column packing	Column temperature	Other drugs analysed	Ref.
0.5	5-Ethyl-5-*p*-tolylbarbituric acid, amylobarbitone or hexobarb	No	3% SP 2250 DB	Isothermal	Primidone, phenobarbitone	5
1.0	Ethyl-*p*-tolylmalonamide	No	3% OV-17	Isothermal	None	6
1.0	None	Yes, trimethylsilyl derivative	1% OV-17	Temperature programme	Primidone, phenobarbitone, phenytoin	7
1.0–5.0	None	Yes, trimethylsilyl derivative	1% OV-17	Temperature programme	Primidone, phenobarbitone, phenytoin	8
0.5	*p*-Methyl-PEMA	Yes, trifluoroacetic anhydride derivative	3% OV-1	Isothermal	None	9
5–100 µl	2-Ethyl-2-*p*-tolylmalonic acid diamide	Yes, trimethylsilyl derivative	3% OV-17	Temperature programme	*p*-Hydroxyphenobarbitone, phenobarbitone, primidone	10
1.0	*p*-Methyl-PEMA	Yes, DMF-DMA derivative	3% OV-225	Isothermal	Carbamazepine	11
1.0	Benzylmalonate methyl ester monoamide	Yes, Regis silylation mix	3% OV-17	Temperature programme	Primidone	12
1.0–2.0	Methylphenobarbitone, methylprimidone, methylphenytoin	Yes, trimethylphenyl ammonium hydroxide	3% OV-17	Temperature programme	Phenytoin, primidone, phenobarbitone, dimethadione, trimethadione, valproate, carbamazepine, ethosuximide	13
1.0	Mesantoin	No	(A) 0.8% SP 1000 (B) 1% SP 1000		Ethosuximide, primidone, phenobarbitone, phenytoin, carbamazepine	14
20 ml urine or cerebrospinal fluid	Docosane	Yes, diazomethane	3% OV-17	Temperature programme	Primidone, phenobarbitone	15
1.0	Methylphenytoin	No	2% SP 2510 DA	Temperature programme	Primidone, phenobarbitone	16

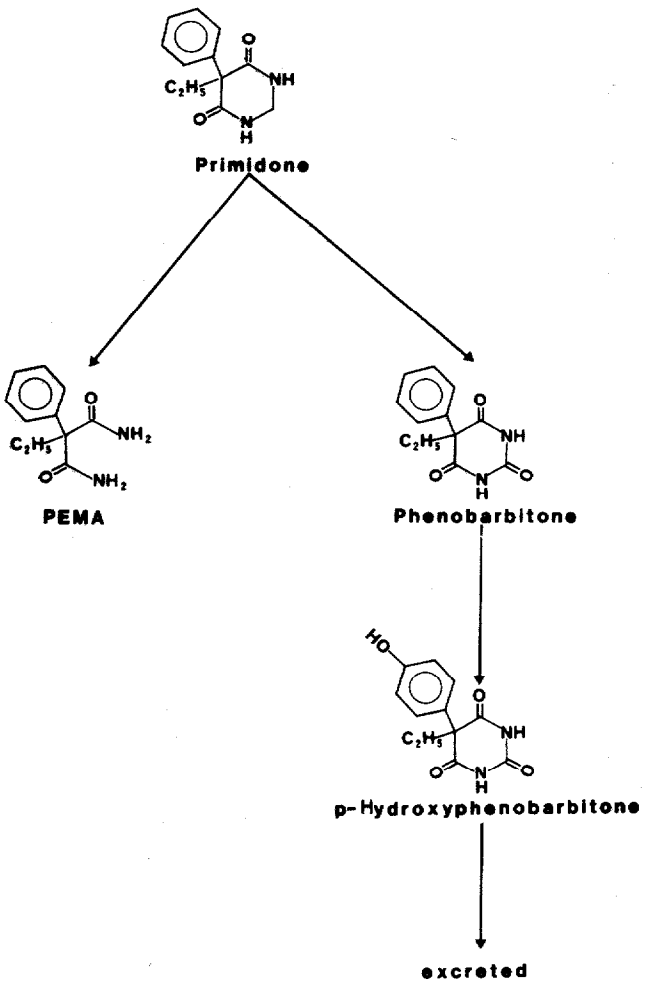

Fig. 1. Major pathways of primidone metabolism in man.

use in the treatment of essential tremor [3,4]. In addition, accurate information about the pharmacokinetics of PEMA is lacking and most investigators have studied PEMA which has been produced from the parent drug rather than administering the metabolite directly.

A variety of gas chromatographic (GC) procedures for the measurement of PEMA in plasma have been reported (see Table I). Most methods combine the measurement of PEMA with other anticonvulsants and more than half of them require formation of derivatives prior to chromatography. In addition, temperature programming is often necessary to achieve separation of the various drugs. Many of the methods have not investigated the possibility of interference from endogenous dietary xanthine derivatives which might co-chromatograph with PEMA. In our experience such interferences are quite common, e.g. in the modification of a rapid method for measuring anticonvulsant drugs reported by Ruth-

erford and Flanagan [17], a C-87 liquid phase is used on which PEMA and caffeine cannot be separated.

High-performance liquid chromatographic (HPLC) methods to measure PEMA are also available, and these normally use reversed-phase systems. In order to separate a wide range of anticonvulsants in a reasonable length of time, it is often necessary either to heat the analytical column [18] or use gradient elution techniques [19].

The present method was developed to measure both plasma and urine concentrations of PEMA in healthy volunteers and epileptic patients after they had been administered a single oral dose of this compound. The plasma concentration data were used to determine the pharmacokinetic parameters of PEMA in both groups [20].

In addition, the method has been used to undertake a long-term survey of PEMA levels in blood samples submitted to our antiepileptic drug-monitoring service from patients whose epilepsy is treated with primidone [21]. The difficulties of interpreting these data are considerable, especially in those patients who are prescribed more than one drug. However, it was important to undertake the study, because several of the cases of toxicity which have been reported in patients who were prescribed primidone have been attributed to elevated plasma concentrations of PEMA. Accumulation of this metabolite is reported to occur particularly in patients with impaired renal function [22,23] and since this laboratory provides an anticonvulsant monitoring service to a large number of hospitals its workload provides sufficient samples to investigate such reports in greater depth.

The present paper provides details of an investigation of GC conditions for the analysis of PEMA and describes a specific, sensitive, accurate and reproducible assay which was suitable for the purposes outlined above.

EXPERIMENTAL

Reagents

The sources of various chemicals used in this work are as follows: dichloroethane (HPLC grade), Rathburn Chemicals (Walkerburn, U.K.); chloroform, acetone and sodium dihydrogen orthophosphate (Analar grade), BDH (Poole, U.K.); 2-ethyl-2-phenylmalonamide monohydrate (PEMA) and 2-ethyl-2-(*p*-tolyl)malonamide (EPTMA), Aldrich (Gillingham, U.K.); caffeine, theophylline, theobromine and paraxanthine, Sigma (Poole, U.K.); phenobarbitone, May and Baker (Dagenham, U.K.); phenytoin, Parke Davis (Eastleigh, U.K.); primidone, ICI (Macclesfield, U.K.); Equine plasma, Gibco Bio-Cult (Uxbridge, U.K.); 0.4 *M* sodium dihydrogen orthophosphate buffer, pH 4.5, BDH; Whatman No. 1 filter papers (solvent washed, see text).

Instrumentation

A Pye 104 Model 24, dual-column gas chromatograph equipped with a flame ionisation detector was used throughout, in conjunction with a Bryans 28000 recorder with 1 mV f.s.d. The temperature at which each column was tested and

the amplifier setting employed are shown in Table II. Argon was used as a carrier gas at a flow-rate of 50–60 ml/min.

Columns

Each column consisted of a coiled glass tube, 1.52 m×2 mm I.D., which was silanised by sucking through a few millilitres of dichlorodimethylsilane, using a water pump. The column was then washed with methanol to remove excess dichlorodimethylsilane and dried in an oven at 120°C prior to packing. Each stationary phase was retained in the columns by plugging the ends with silanised glass wool (Chromatography Services, Merseyside, U.K.). All column packings were prepared at a 3% loading unless otherwise stated in Table II. Before use, the columns were conditioned overnight at 250–260°C with the carrier gas flowing.

The CDMS/WG11 packing was made by first dissolving 0.4 g of CDMS and 0.1 g of WG11 in 100 ml of dichloroethane, then 10 g of Chromosorb W HP 100–120 mesh (Perkin-Elmer, Beaconsfield, U.K.) were added, and the mixture was stirred before being left to settle. Any fines which were floating were then removed and the mixture was stirred again prior to vacuum filtering through a Whatman No. 50 filter paper.

Qualitative analysis

The sensitivity of each column was tested by injecting standard solutions of each drug under the conditions shown in Table II.

Quantitative analysis

Two stock solutions were prepared which contained PEMA at a concentration of 1 g/l in both ethanol and water. Warming was necessary in order to dissolve the PEMA in water. EPTMA, the internal standard, was prepared at a concentration of 1 g/l in ethanol. Aqueous calibration standards were prepared using the aqueous stock solution at the following concentrations 40, 20, 15, 10, 5 and 2.5 mg/l. Plasma calibration standards were prepared in a similar way and at the same concentrations by adding the ethanolic stock solution to Equine plasma.

Quality controls

The quality controls were prepared by diluting the ethanolic stock solution of PEMA in Equine plasma as to make 10 ml of 7, 12 and 25 mg/l quality control solutions. After mixing thoroughly they were divided into 1-ml aliquots and stored at −20°C.

Extraction procedure

A flow diagram showing the extraction method for PEMA is given in Fig. 2. Samples, quality controls and aqueous calibration standards were treated in an identical manner. Peak heights of drug and internal standard were measured from the apex of the peak to a tangent skim of the baseline and the PEMA/EPTMA peak-height ratio was calculated. A graph relating peak-height ratio to drug concentration was constructed from the aqueous calibration standards and used to calculate the PEMA concentrations of the quality controls and samples.

TABLE II

SUMMARY OF RETENTION TIMES FOR COMMONLY PRESCRIBED ANTICONVULSANTS AND SOME ENDOGENOUS COMPOUNDS ON THE VARIOUS LIQUID PHASES ASSESSED

Compound	Concentration in chloroform (mg/l)	Retention time (min)									
		OV-1	OV-7	OV-17	OV-210	OV-225	CDMS	Poly A	10% Apiezon L	2% SP 2510 DA	CDMS/WG11
PEMA	(80)20	1.4	1.8	2.4	3.7	3.2	1.9	2.5	2.4	2.2	3.8
Internal standard (EPTMA)	30	1.4	2.5	3.3	5.2	4.1	2.5	3.3	3.6	2.7	5.0
Phenobarbitone	50	1.5	2.1	2.3	3.6	3.6	3.5	5.9	3.5	5.5	7.8
Phenytoin	50	-	8.3	12.9	14.8	18.5	18.7	37.0	11.6	18.7	46.0
Primidone	25	-	6.2	10.0	12.6	15.4	9.6	18.7	7.0	11.7	23.6
Carbamazepine	25	-	7.4	2.6	15.8	10.7	5.8	3.0	-	5.9	11.2
Caffeine	50	0.8	1.4	1.7	2.1	1.5	0.8	1.0	2.6	0.9	1.2
Theophylline	50	-	2.6	4.0	4.4	5.2	4.1	10.5	3.6	5.0	9.2
Theobromine	50	1.4	1.6	2.3	3.6	2.8	1.6	1.9	2.7	1.9	3.0
1,7-Dimethylxanthine	50	-	1.7	2.0	4.0	3.0	2.3	3.0	3.2	2.2	4.3
Cholesterol	100	-	47.0	35.0	29.0	14.3	6.7	-	-	15.4	12.4
Column temperature (°C)		180	218	222	190	235	243	228	235	222	219
Attenuation		$2 \cdot 10^2$	$5 \cdot 10^2$	$5 \cdot 10^2$	$2 \cdot 10^2$	$5 \cdot 10^2$	$2 \cdot 10^2$	$5 \cdot 10^2$	$5 \cdot 10^2$	$10 \cdot 10^2$	$5 \cdot 10^2$

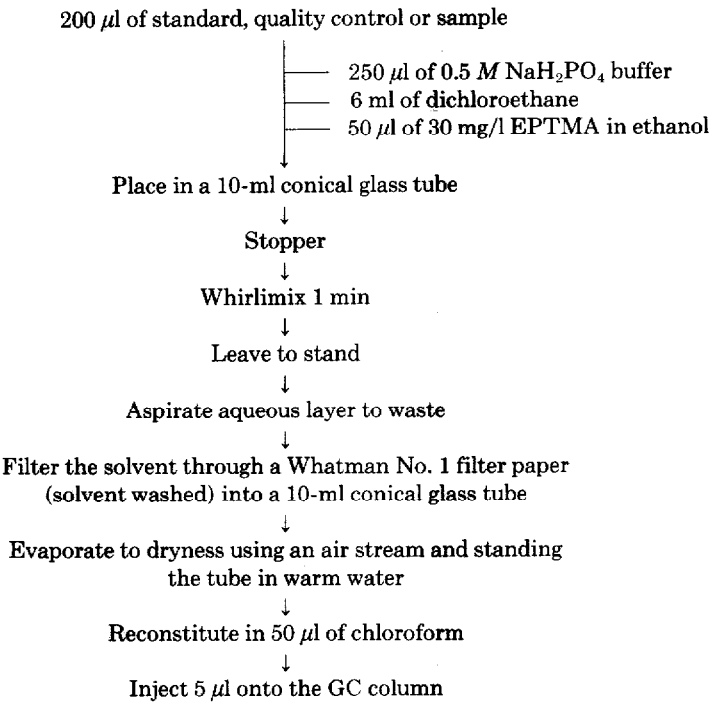

Fig. 2. Flow diagram showing the extraction method for PEMA.

RESULTS AND DISCUSSION

A rectilinear relationship exists between the PEMA/EPTMA peak-height ratio and PEMA concentration ($r=0.96$). The quality control values were correct within the reproducibility of the method when calculated from either water or equine standards.

Recovery experiments

Calibration graphs which had been prepared from both the aqueous and plasma standards were superimposable and for simplicity aqueous standards were used for routine quantitation of PEMA samples. Since the apparent extraction efficiency from water and plasma was identical, it was not necessary to apply a recovery factor to these results.

The processed water standards were compared with solutions which had been prepared by weighing PEMA and EPTMA into chloroform. The concentration of PEMA in these solutions had been adjusted to compensate for the 4:1 concentration of PEMA that occurs when samples and standards are carried through the extraction procedure. The apparent recovery was calculated to be 75%.

Preparation and choice of column

With the exception of the commercially prepared packing (SP 2510 DA) and the CDMS/WG11, the packings were all prepared by the same evaporation method

[24]. Although the precise loading of the CDMS/WG11 was not determined, and is uncertain, since it was prepared by a filtration technique, the packing is reproducible. The CDMS/WG11 column was stable for several months; it was discarded once the resolution of PEMA and EPTMA decreased and the peak shapes broadened.

All of the columns except SP 2510 DA and CDMS/WG11 were rejected after preliminary testing (Table II), because of poor sensitivity or poor resolution of PEMA from phenobarbitone or the xanthine derivatives. Since phenobarbitone occurs in all specimens from patients who are prescribed primidone it must be adequately separated from PEMA. Initially the SP 2510 DA phase was used for the analysis, but an additional peak was present in the traces and column life at the operating temperature used was short. The additional peak interfered with quantitation since it eluted in front of PEMA, but was not completely resolved. Also, it was not possible to use the extraction procedure outlined in Fig. 2 when using the SP 2510 DA column because the calibration was not rectilinear. However a linear calibration could be obtained by direct extraction of 100 μl of sample or standard (in duplicate) into chloroform using EPTMA (10 mg/l) as an internal standard.

The CDMS/WG11 phase produced the most satisfactory results since its sensitivity was adequate and not only was the column performance stable from day to day, but its lifetime was several months. In addition, no endogenous compounds which co-chromatographed with either PEMA or the internal standard were extracted, and no drugs have been found to interfere with the assay. EPTMA was chosen as the internal standard because it possesses similar extraction and chromatographic characteristics as PEMA. The CDMS/WG11 column produced an excellent separation between the two analogues (Fig. 3), and its only disadvantage was the need to wait 15 min between injections to allow primidone to elute when assaying clinical samples. Phenytoin eluted later (approx. 46 min) but only caused a problem when the phenytoin level was high.

Reproducibility

Two samples were prepared by spiking plasma with PEMA at concentrations of 15 and 2.5 mg/l. These were assayed ten times each within the same batch of analyses and the results are summarised below. For the 15 mg/l sample the standard deviation (S.D.) was 0.0515 and the coefficient of variation (C.V.) 2.62%. For the 2.5 mg/l sample the S.D. was 0.01664 and the C.V. 5.07%. The accumulated results of the three quality control specimens which were assayed as part of each batch of analyses have been used to calculate the between-assay precision. For the 7.0 mg/l quality control: S.D. = 0.201; $n = 80$; C.V. = 2.84%. For the 12.0 mg/l quality control: S.D. = 0.319; $n = 79$; C.V. = 2.67%. For the 25.0 mg/l quality control: S.D. = 1.10; $n = 82$; C.V. = 4.44%.

Extraction procedure

The extraction method employs a 4:1 concentration step and this produced adequate sensitivity to measure PEMA not only in clinical samples but also fol-

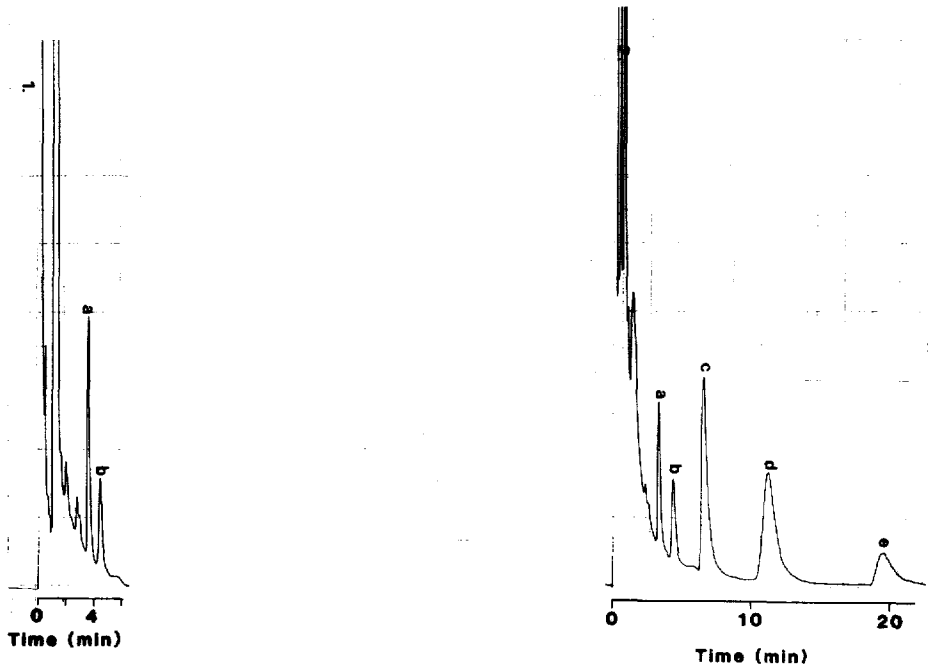

Fig. 3. Gas chromatograms of (1) extracted water standard and (2) extracted sample from a patient receiving primidone therapy using the CDMS/WG11 stationary phase. Peaks: a = PEMA; b = internal standard (30 mg/l EPTMA); c = phenobarbitone; d = cholesterol; e = primidone.

lowing single therapeutic doses. During the initial work with the SP 2510 DA phase only a 2:1 concentration step could be used.

The Whatman No. 1 filter papers were washed twice in dichloroethane prior to use in order to eliminate interfering peaks. This was done by soaking the filter papers for 30 min in a beaker of dichloroethane. The solvent was then discarded, fresh solvent added and the papers were again soaked for 30 min. After discarding the dichloroethane, the filter papers were left to dry in a fume cupboard.

Both urine and plasma samples can be assayed by this method. Urine concentrations of PEMA are greater than those in plasma so calibration standards in the range 10–80 mg/l were prepared by spiking PEMA into blank urine. The extraction procedure for urine is as outlined in Fig. 2 except that a smaller (100 μl) volume was assayed in duplicate.

Unfortunately the chromatography time for plasma samples is quite long since it is necessary to wait between injections for cholesterol and also, in the case of clinical samples, for primidone to elute (see Fig. 3, a typical sample trace).

Internal standard

The internal standard was suggested by other workers in this field and is readily available [25].

Patient samples

This method has been in regular use for five years and during this time the PEMA concentration has been determined in the majority of patient samples

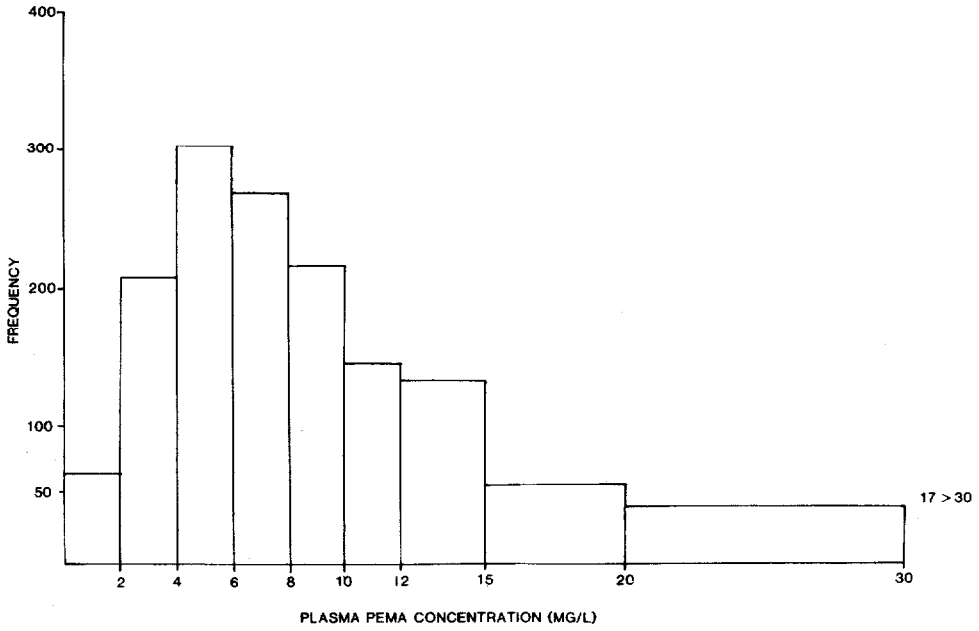

Fig. 4. Distribution of PEMA concentrations in 1461 samples from patients receiving primidone therapy.

submitted to us for primidone analysis. Fig. 4 shows the distribution of PEMA concentrations in 1461 samples. Some of these results will be repeat assays on the same patient as some epileptics have their anticonvulsants drug levels regularly monitored.

Plasma PEMA concentrations above 20 mg/l could be toxic (the majority of PEMA concentrations are less than 15 mg/l) and elevated levels were found in 58 samples taken from 46 different patients. A further study which hopes to identify the factors that cause excessive accumulation of PEMA is under way. To date it has been shown that the majority of patients with raised PEMA levels also have raised primidone and/or phenobarbitone concentrations in their plasma but raised primidone and/or phenobarbitone levels do not necessarily result in elevated PEMA concentrations.

CONCLUSIONS

Using the CDMS/WG11 column and the bulk extraction method outlined it was possible to analyse PEMA in samples from patients receiving primidone. The method was also used to measure both plasma and urine PEMA concentrations in normal volunteers and epileptic volunteers who had been dosed with PEMA itself [20]. The results obtained by the present method correlated well with those from a second laboratory which used GC, and with those obtained by an HPLC method which is under development in this laboratory.

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REFERENCES

1 B.B. Gallagher and I.P. Baumel, in D.M. Woodbury, J.K. Penry and R.P. Schmidt (Editors), Antiepileptic Drugs, Raven Press, New York, 1972, Ch. 35, p. 353.
2 B.B. Gallagher and I.P. Baumel, in D.M. Woodbury , J.K. Penry and R.P. Schmidt (Editors), Antiepileptic Drugs, Raven Press, New York, 1972, Ch. 36, p. 357.
3 M.D. O'Brien, A.R. Upton and P.A. Toseland, Br. Med. J., 282 (1981) 178.
4 S. Calzetti, L.J. Findley, F. Pisani and A. Richens, J. Neurol. Neurosurg. Psychiatry, 44 (1981) 932.
5 R.J. Hunt and K.W. Miller, Drug Metab. Dispos., 6 (1978) 75.
6 D. Haidukewych and E.A. Rodin, Clin. Chem., 26 (1980) 1537.
7 I.P. Baumel, B.B. Gallagher and R.H. Mattson, Arch. Neurol., 27 (1972) 34.
8 B.B. Gallagher, I.P. Baumel and R.H. Mattson, Neurology, 22 (1972) 1186.
9 J.E. Wallace, H.E. Hamilton, E.L. Shimek and H.A. Schewertner, Anal. Chem., 49 (1977) 1969.
10 H. Nau, D. Jesdinsky and W. Wittfoht, J. Chromatogr., 182 (1980) 71.
11 R.J. Perchalski and B.J. Wilder, J. Chromatogr., 145 (1978) 97.
12 C.J. Least, G.F. Johnson and H.M. Solomon, Clin. Chem., 21 (1975) 1658.
13 W. Loscher and W. Gobel, Epilepsia, 19 (1978) 463.
14 R. Heipertz, H. Pilz and K. Eickhoff, Clin. Chim. Acta, 77 (1977) 307.
15 M.W. Couch, M. Greer and C.M. Williams, J. Chromatogr., 87 (1973) 559.
16 R.A. Yeary, Am. J. Vet. Res., 11 (1980) 1643.
17 D.M. Rutherford and R.J. Flanagan, J. Chromatogr., 157 (1978) 311.
18 H.M. Neels, J.A. Totte, R.M. Verkerk, A.J. Vlietinck and S.L. Scharpe, J. Clin. Chem. Clin. Biochem., 21 (1983) 295.
19 N. Wad, J. Chromatogr., 305 (1984) 127.
20 P.R. Cotterell, J.M. Streete, D.J. Berry, H. Schafer, F. Pisani, E. Perucca and A. Richens, Epilepsia, 23 (1982) 307.
21 J.M. Streete, D.J. Berry, L.I. Pettit and J.E. Newbery, Ther. Drug Monit., 8 (1986) 161.
22 E.L. Stern, Ann. Neurol., 2 (1977) 356.
23 R. Heipertz, A. Guthoff and W. Bernhardt, J. Neurol., 221 (1979) 101.
24 D.J. Berry, J. Chromatogr., 86 (1973) 89.
25 C.E. Pippenger, J.K. Penry and H. Kutt (Editors), Antiepileptic Drugs: Quantitative Analysis and Interpretation, Raven Press, New York, 1978, Appendix IIB, p. 359.