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Note

Simultaneous assay of methylphenobarbital and phenobarbital in plasma using gas chromatography—mass spectrometry with selected ion monitoring

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Methylphenobarbital (mephobarbital; MPB) has been used as an anticonvulsant since 1932 [1]. The clinical pharmacokinetics and metabolic fate of the drug have been incompletely documented [2]. It has been long recognized that the drug is metabolically N-demethylated to phenobarbital (PB) [3]. There has been some study of the plasma levels of MPB [4] and PB [4, 5] which occur in patients taking MPB, and it is known that steady-state plasma levels of PB exceed those of MPB by a factor of 7—10 [6] or even more [4].

Plasma levels of barbiturates are frequently determined by gas—liquid chromatography (GLC) with on-column methylation [7] or by a homogeneous enzyme immunoassay technique [7]. Neither of these methods discriminates between MPB and PB. Specific assays for MPB and PB have been achieved by GLC involving on-column butylation [8], by direct chemical ionization mass spectrometry (MS) [9], and by a selected ion monitoring gas chromatography (GC)—MS technique [4]. Although there have been several reports of the use of high-performance liquid chromatographic techniques for barbiturate analysis (e.g. ref. 7), we are aware of only one report in which both MPB and PB were included [10]. None of these methods fulfilled the requirements of our proposed pharmacokinetic studies with MPB [11], for which a simultaneous assay for MPB and PB with good precision, low detection limits and convenience for coping with large sample numbers was desired. The present method met these criteria.

MATERIALS AND METHODS

Chemicals and reagents

MPB and PB were purchased from Applied Science Labs. (State College, PA, U.S.A.) and the internal standard, 5-ethyl-5-(4-methylphenyl)-barbituric acid

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(*p*-tolylbarbituric acid; TB) was obtained from Aldrich (Milwaukee, WI, U.S.A.). Stock solutions of each barbituric acid in methanol were prepared at a concentration of 50 mg l^{-1}. N,N-Dimethylacetamide and tetramethylammonium hydroxide were from Sigma (St. Louis, MO, U.S.A.) and 1-iodopropane from Aldrich. Chloroform was analytical reagent grade, and was distilled before use.

Extraction and derivatization

Exactly 100 μl of the methanolic internal standard (TB) solution were dispensed into the assay tube (Pyrex tube, 150 × 20 mm with Teflon-lined screw cap), and the methanol was evaporated with a nitrogen stream. Plasma (0.1 —1.0 ml) was added, followed by 0.3 ml hydrochloric acid (0.2 *M*) and 5.0 ml chloroform. After shaking (2 min) and centrifuging (2 min at 1000 *g*), the aqueous layer was aspirated to waste, and the chloroform phase poured carefully into a clean test tube with a conical bottom. The chloroform was evaporated to dryness (nitrogen stream and water bath at 60°C), and the residue derivatized 10—15 min prior to chromatography. Derivatization was based on the method of Greeley [12], and was effected by taking the residue up in N,N-dimethylacetamide (40 μl), adding tetramethylammonium hydroxide (5 μl of a 20% w/v solution in methanol) and 1-iodopropane (10 μl). After 10—15 min standing, the precipitate (tetramethylammonium iodide) was centrifuged to a pellet, and 1—5 μl of the clear supernatant was injected into the GC—MS system.

GC—MS analysis

A Finnigan Model 3300F GC—MS system with a Model 6110 data system was used in these studies. The gas chromatograph was fitted with a 1.5 m × 2 mm I.D. glass column packed with 3% OV-101 on 80—100 mesh Gas-Chrom Q (Applied Science Labs.). The injector, glass jet separator and glass-lined transfer line were all at 250°C, and the column oven at 200°C. Helium flow-rate was 25 ml min^{-1}. The mass spectrometer was operated in the electron impact ionization mode, and the ions at *m/z* 146 and 160 were monitored. The ion source filament was left on throughout the run, to maintain source conditions as stable as possible.

Quantitation

Standard calibration graphs were obtained by the analysis of 1-ml aliquots of drug-free human plasma, to which had been added known amounts of MPB and PB. Peak heights were measured in preference to peak areas, as we attained better precision by this approach, in agreement with the recommendations of Millard [13]. Precision and detection limits were assessed by standard procedures. Extraction recovery was assessed by comparing the peak height ratios obtained for a set of plasma standards (5 mg l^{-1}) with those applying when 5.0 μg of each analyte were added to a tube containing TB which had been extracted from blank plasma. This procedure compared the extracted and unextracted analytes, both relative to the same quantity of (extracted) internal standard.

RESULTS

The electron impact spectra of the propylated derivatives of the three barbi-

turic acids are shown in Fig. 1. Similar spectra have been reported and interpreted by others [14,15]. The ions chosen for selected ion monitoring were the base peaks in each spectrum.

Typical selected ion chromatograms for blank plasma and for a 1.0 mg l^{-1} plasma standard are shown in Fig. 2. It was possible to inject samples at approximately 6-min intervals, as no responses for the monitored ions were observed after the TB peak had eluted. Processing of plasma standards yielded excellent linear calibration curves for each barbiturate, the correlation coefficient

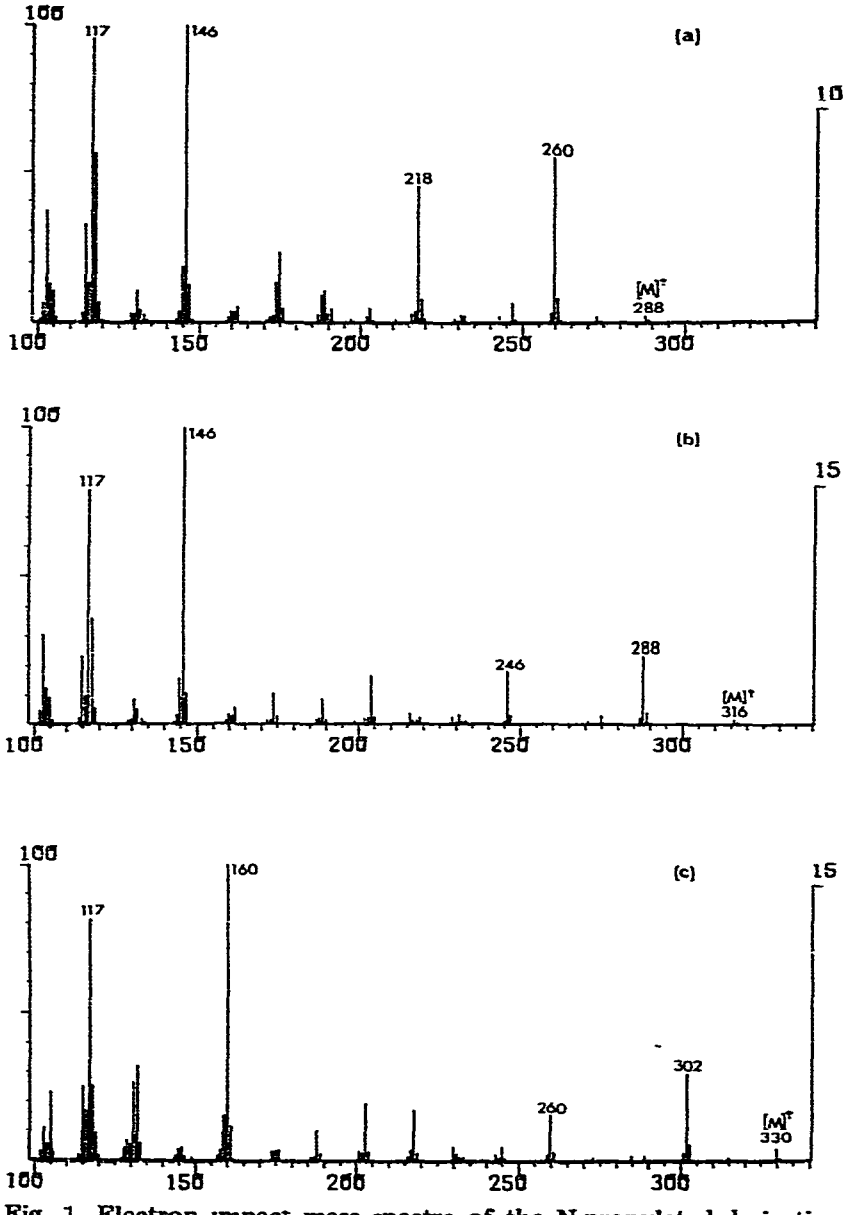

Fig. 1. Electron impact mass spectra of the N-propylated derivatives of (a) methylphenobarbital, (b) phenobarbital and (c) *p*-tolylbarbituric acid.

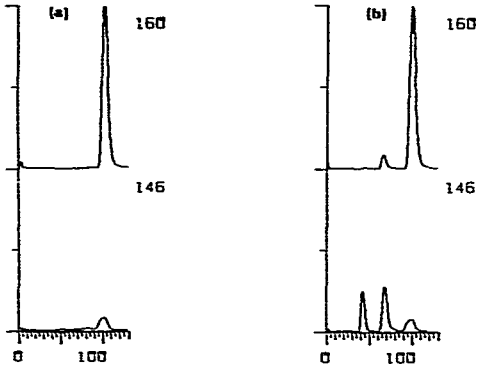

Fig. 2. Selected ion profiles for *m/z* 146 and 160 for (a) blank plasma and (b) plasma standard with both drugs at 1 mg l^{-1}. The scale on the abscissa represents points at which the ion current was sampled (actually 2-sec intervals).

always being greater than 0.99. The within-batch precision was very good, giving a coefficient of variation of less than 4% for each drug, with a standard error of the mean of 2% (n = 9). Thus assay of a 5 mg l^{-1} standard gave a value of 5.0 ± 0.18 (S.D.; n = 9), while at 1 mg l^{-1} the result was 1.0 ± 0.04 (S.D.; n = 9). However it was noted that minor variations in the slope of the calibration graph did occur from day to day. This effect has also been documented by Millard [13], and is a function of mass spectrometer tuning and ion source stability. Knowing that the calibrations were consistently linear, it was deemed preferable to measure concentrations in a day's assays by reference to a single point (5 mg l^{-1}) standard, multiples of which were extracted with the batch. As a control on instrumental stability, an injection was made from one of these standards after every three sample injections. Concentrations of MPB and PB were then calculated by reference to the mean of at least 9 such standard injections. The precision figures quoted above were obtained in this manner, indicating good instrument stability over the several hours necessary to collect the data.

Extraction recovery was calculated as 97% for MPB and 92% for PB. The lower limit of detection for both drugs, using a 1.0-ml plasma sample, was approximately 20 ng ml^{-1}.

An illustration of the application of the method to the single-dose pharmacokinetics of MPB is given in Fig. 3, which shows the time course for both MPB and PB following a single 100-mg intravenous dose of MPB in one volunteer. MPB (0.16 mg l^{-1}) and PB (0.17 mg l^{-1}) were still measurable after 9 days, and the elimination half-life of MPB was calculated as 64.2 h.

DISCUSSION

The use of GC—MS with selected ion monitoring for the determination of these barbiturates has been reported recently by Kupferberg and Longacre-Shaw [4]. Our method differs from theirs principally in regard to the derivatization technique, and the choice of ions for monitoring. The alkylation procedure of Kupferberg and Longacre-Shaw [4], which involved heating the samples for 30 min seemed less satisfactory than the milder method of Greeley [12]. While the former method is desirable for derivatization of relatively non-acidic

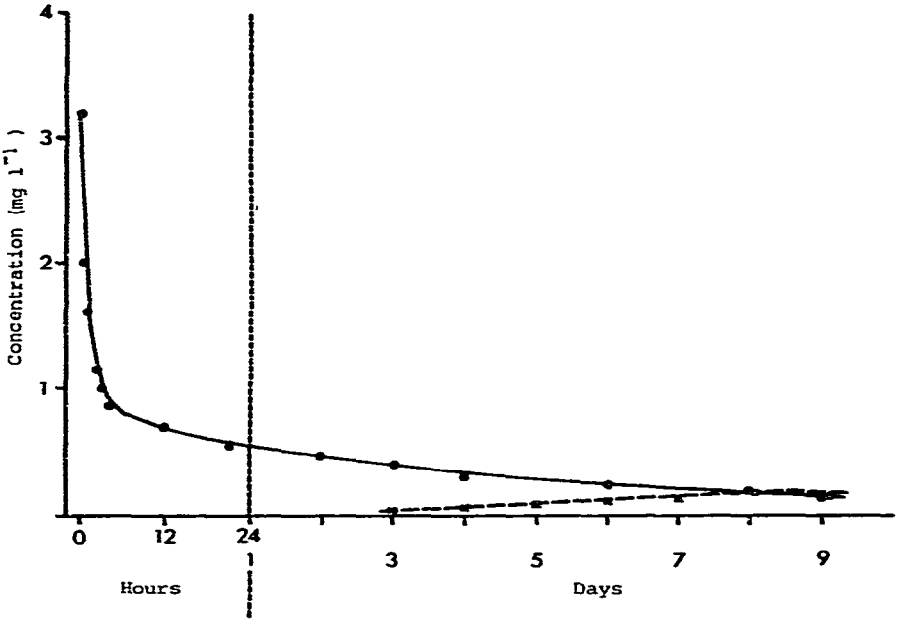

Fig. 3. Plasma concentrations of MPB (•——•) and PB (x– – –x) following a 100-mg intravenous dose of MPB in a volunteer. The vertical dotted line marks a change in the abscissa scale from hours to days. No PB was detected before day 3.

protons (such as that at N^1 in the hydantoin anticonvulsants [16], it may cause problems with the more hydrolytically labile barbiturate ring, the base catalysed fission of which has been carefully studied [17]. Although we sought to standardize the time from derivatization to chromatographing at 15 min, no significant degradation was apparent until more than two hours had passed. Unwanted hydrolytic reactions were also reduced by keeping the quantity of tetramethylammonium hydroxide catalyst to a minimum. Our selection of the base peak ions (*m/z* 146 and 160) for monitoring was based on our need for a highly sensitive assay. We observed that the $(M-28)^+$ fragment was the base peak for N-methyl and N-ethyl barbiturates, in agreement with Kapetanović and Kupferberg [18]. However for the N-propyl derivatives which we used on account of their satisfactory chromatographic resolution, the $(M-28)^+$ ion was only 25—50% of the base peak (Fig. 1). The observation that increasing chain length in the N-alkyl substituent can promote alternative fragmentation processes in the mass spectra of barbiturates has been documented previously [19]. Although the ions which we selected may present greater potential for interference [13] than the higher mass, but less abundant, ions, we have not encountered interfering compounds in biological samples to date. These problems could presumably be largely overcome by monitoring quasi-molecular ions in the chemical ionization mode [9], but we lacked the facilities to investigate this question.

The present method was developed primarily for the study of single-dose pharmacokinetics of MPB. Its application is illustrated here in the case of a single 100-mg intravenous dose to a volunteer. The method has also been ap-

plied to a more detailed investigation [11] of the kinetics of MPB than was achieved [6] with our earlier GLC technique [8]. The method is also applicable to studies in neonates, or for analysis of trace quantities of MPB and PB in specimens such as saliva and breast milk, for which similar methods have recently been reported for the related anticonvulsant, primidone [20].

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