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Metabolic studies with phenobarbitone, primidone and their N-alkyl derivatives: quantification of substrates and metabolites using chemical ionization gas chromatography–mass spectrometry

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SUMMARY

Metabolic studies with phenobarbitone, primidone and some of their N-alkyl derivatives required the concurrent assay of any mixture of these substrates (twelve compounds) and their major metabolites (an additional twenty-two compounds) in urine. The method described in the present report met this requirement by incorporating two complementary derivatization techniques into a gas chromatographic–mass spectrometric (GC–MS) assay procedure Following hydrolysis of conjugates with β-glucuronidase, urine samples were extracted with ethyl acetate (3×5 ml). The combined extracts were dried over sodium sulphate, divided into two equal portions, and the solvent was removed. One residue was derivatized by propylation using 1-iodopropane with base catalysis. The other residue was silylated using methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide. The derivatives in each case were analysed by GC–MS, using temperature-programmed packed-column GC and chemical ionization MS. Mass spectra were acquired over an appropriate mass range, and peak areas for the compounds of interest were determined from specific mass chromatograms. Satisfactory precision, accuracy, specificity and sensitivity were obtained for all analytes. All compounds produced satisfactory derivatives by at least one proce-

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dure; twelve compounds could be analysed by both techniques. The method illustrates the utility of chemical ionization GC–MS for the simultaneous quantitative analysis of multiple related analytes in complex biological samples.

INTRODUCTION

We have previously reported widely differing extents of metabolic hydroxylation in the 5-phenyl ring of the structurally related anticonvulsants methylphenobarbital (MPB; **1b**) and primidone (PRM; **2a**) (Fig. 1). The *p*-hydroxy metabolites of MPB and PRM, compounds **3b** and **4a**, respectively, account in human urine for ~40% [1] and <1% [2] of single oral doses of the drugs. This compares with the intermediate value of approximately 20% for the corresponding metabolite (**3a**) of phenobarbitone (PB; **1a**) [3].

We have undertaken metabolic studies with compounds **1a–f** and **2a–f** to seek correlations between physicochemical parameters of these twelve substrates and their metabolic fates. The syntheses of these two series of analogues have been reported previously [4].

Metabolites of these compounds were predicted to include, inter alia, the *p*-hydroxy derivatives **3a–f** and **4a–f**, O-methylcatechols (**5a–f**) from the barbiturates and 2-ethyl-2-phenylmalondiamides (**6a–f**) from the corresponding primidones (**2a–f**). Products of N-dealkylation, and of 2-oxidation of **2** to yield **1**, were also anticipated. The O-methylcatechols of the primidones were considered unlikely to be quantitatively important, on account of the relatively low extent of phenyl ring hydroxylation observed with these compounds [2].

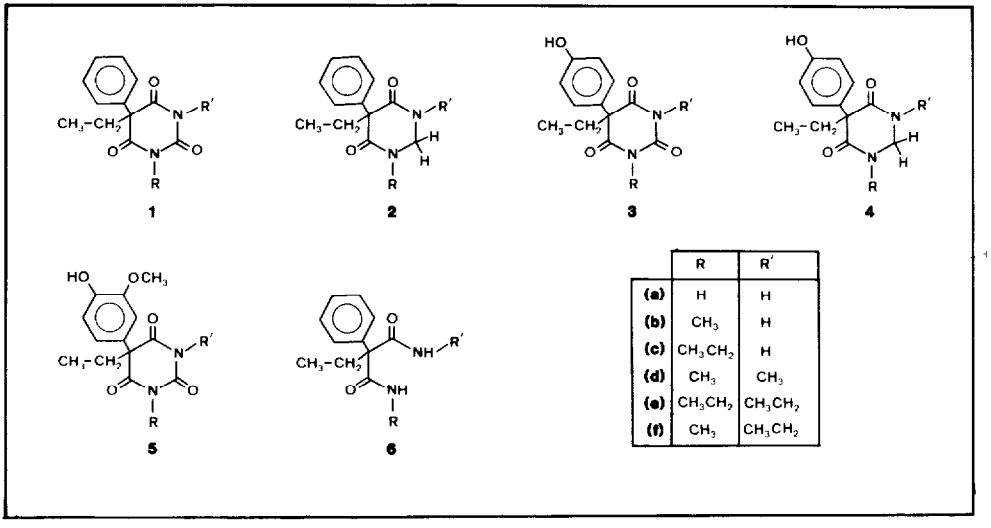

Fig. 1. Structures of the substrates and metabolites.

Up to eighteen of these potential metabolites could in theory arise from some of the compounds **1** or **2**, by sequential metabolic events. The metabolic studies, therefore, effectively imposed a requirement for the specific quantification of all thirty-six compounds in the presence of any combination of the others.

This paper describes the gas chromatographic–mass spectrometric (GC–MS) procedure which we have developed to meet this requirement. The preferred approach to quantification by GC–MS, using specific ion monitoring and stable isotope-labelled internal standards [5], was impractical for the present analyses. Synthesis of so many labelled analogues was not feasible, and hardware and software limitations of our data system prohibited monitoring a sufficient number of selected ions for this approach. Satisfactory procedures for quantification have been achieved using chemical ionization GC–MS and specific mass chromatogram retrieval from data files acquired as full scan mass spectra.

EXPERIMENTAL

Drugs and metabolites

PB (**1a**, B.P. grade) was obtained from Queensland Ethicals (Brisbane, Australia). MPB (**1b**, B.P.) was a gift from Sterling Pharmaceuticals (Ermington, Australia). PRM (**2a**, B.P.), 2-ethyl-2-phenylmalonyldiamide (PEMA; **6a**), N-methyl-PEMA (**6b**) and N-ethyl-PEMA (**6c**) were kindly donated by ICI Labs. (Melbourne, Australia).

The other N-alkylbarbiturates (**1c–f**) [4], N-alkylprimidones (**2b–f**) [4], O-methylcatechols (**5a–e**) [6] and malonyldiamides (**6d–f**) were synthesized in our laboratories, recrystallized to analytical purity and verified by GC–MS and NMR spectroscopy.

The *p*-hydroxy derivatives of compounds **1** and **2** (**3a–e** and **4a–f** respectively) were synthesized in our laboratories,using standard techniques of which only an outline is given here. The *p*-hydroxy derivative of phenobarbital (**3a**) was synthesized by Butler [7] following the classical route of nitration, reduction, diazotization and hydrolysis. We have adopted this same sequence for synthesis of the eleven *p*-hydroxy compounds noted above. Butler observed that the nitration step yielded the *m*-nitro derivative as the major product, while yields of the desired *p*-nitro isomers were relatively low. We are indebted to a colleague, Dr. H.E. Kunze, who during earlier studies on MPB [1] noted that the relative proportion of the desired *p*-nitro isomer was greatly increased if the reaction was carried out at elevated temperatures (up to 100 °C) rather than the conventional 0–5 °C; the undesired di-nitro derivatives were very minor by-products, and their formation was minimized by rapid addition of nitrating acid to the barbiturate solution, using only one molar equivalent of nitric acid. The nitro compounds were carried through the remaining synthetic steps as mixtures, and separation of the *m*-and *p*-isomers of the final hydroxy

compounds was effected by preparative high-performance liquid chromatography (HPLC). Products were finally purified by crystallization. All of the required *p*-hydroxy metabolites were prepared by this procedure except **3f**, whose synthesis was confirmed by mass spectral data, but which we were unable to crystallize. We should note that sufficient quantities of the purified hydroxy compounds were obtained to permit characterization and establishment and calibration of analytical methods, but yields were quite low. It seems probable that somewhat better yields could be obtained by the more recent technique involving nitration with nitronium tetrafluoroborate, which produced predominantly the *p*-nitro product in the case of PB [8]; we were unaware of this procedure when undertaking our synthetic work.

Chemicals and reagents

N,N-Dimethylacetamide and β-glucuronidase (EC 3.2.1.31, from *Helix pomatia*, type H-2) were purchased from Sigma (St. Louis, MO, U.S.A.). Tetramethylammonium hydroxide (24 g per 100 ml methanol) was obtained from Pfaltz and Bauer (Stamford, CT, U.S.A.). 1-Iodopropane and the internal standard, 4′-methylprimidone (TPRM), were purchased from Aldrich (Milwaukee, WI, U.S.A.). N-Methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) was from Regis (Morton Grove, IL, U.S.A.). All other chemicals were of analytical reagent grade.

Metabolic studies

Single oral doses (up to 300 mg) of the barbiturates and primidones were administered to informed, consenting healthy adult male volunteers, and all urine was collected daily for periods of seven to twelve days following each dose. Urine volumes were recorded and aliquots stored at −20 °C until analysed.

Assay procedure

The internal standard (TPRM, 10 μg) was added to each assay tube (15-ml Pyrex tubes with PTFE-lined screw caps) in methanolic solution, and the solvent was evaporated. Aliquots of urine (0.1–1.0 ml) were dispensed, followed by 1 ml acetate buffer (0.2 *M*, pH 5.5) and β-glucuronidase (1000 Fishman units). After incubation at 37 °C for at least 4 h, the solution was extracted with three 5-ml aliquots of ethyl acetate. The extracts were pooled, dried over anhydrous sodium sulphate and then divided into two equal parts (A and B). Solution A was evaporated just to dryness at 40 °C under a stream of air. The residue was taken up in N,N-dimethylacetamide (50 μl), then 15 μl tetramethylammonium hydroxide and, within a few seconds, 15 μl 1-iodopropane were added. Within 10 min the sample was centrifuged briefly at 1000 *g*, and 0.5–5.0 μl of the clear supernatant was injected into the GC–MS system.

To solution B was added N,N-dimethylacetamide (100 μl) and the solution was concentrated under an air stream at 40 °C to a volume of approximately

50 μl. This was mixed with MTBSTFA (50 μl) and warmed to 60 °C for 20 min. The resulting derivatives were shown to be stable for at least 24 h at room temperature. Again 0.5–5.0 μl of the solution of derivatives was injected into the GC–MS system.

Preparation of standards

Calibration standards specific to each study compound were prepared by dispensing into assay tubes, as methanolic solutions, appropriate quantities of each of the reference standards, which could conceivably arise from that substrate, and the internal standard. Following evaporation of the methanol, 1 ml of drug-free urine was added, the tube vortexed briefly and then assayed by the above procedure. With some experience, it was possible to adjust the concentration range of each standard to cover appropriately the range encountered in study samples. However, as will be detailed below, a single-point calibration was used for large batches of assays once the procedure had been adequately validated.

Gas chromatographic–mass spectrometric conditions

A Finnigan 3300F instrument, coupled to a Finnigan 6110 data system, was used for all GC–MS studies. The instrument was fitted with a 1.6 m × 2 mm I.D. glass column packed with 3% OV-101 on Gas Chrom Q, 100–120 mesh (Applied Science Labs., State College, PA, U.S.A.). The carrier gas was methane (18–20 ml/min), which also served as the chemical ionization reagent gas. The column temperature was routinely programmed from 130 to 280 °C at 6 °C/min and the temperature programme and data acquisition were started 1 min after injection. The mass spectrometer was operated in the chemical ionization mode, with source pressure of 1 Torr. Mass spectra were acquired repetitively over the scan range 150–600 daltons, which resulted in a scan time of 4 s. Data collection was continued for up to 30 min following an injection. For urine extracts from some study compounds, abbreviated temperature programmes and mass scan ranges were used resulting in slightly shorter mass spectral scan times. Specific mass chromatograms were recalled from the data files, and peak areas for compounds of interest were measured by standard data system procedures. Calibration curves for each compound were constructed by linear regression analysis of peak-area ratio versus concentration over relevant ranges of concentration. Coefficients of determination for the calibration curves routinely exceeded 0.99.

RESULTS AND DISCUSSION

The selection of study compounds in this work was based on metabolic considerations rather than on analytical convenience; the analytical difficulties proved to be formidable. Thus while the sets of compounds **1–6** have obvious

structural similarities, they encompass a broad spectrum of chemical and physicochemical properties. For example, the substrates span almost 3 (log) units of log P values; the substrates and metabolites range from moderately acidic to neutral; some of the (fully alkylated) compounds are moderately volatile while others are quite non-volatile; and the lability to base-catalysed ring-opening reactions increases sharply when the barbiturate ring is N-alkylated [9]. The simultaneous assay of the individual components of mixtures of up to eighteen of these related compounds with high specificity could not be readily achieved with any technique other than GC–MS.

In a sense the current method is an extension of a much simpler procedure for assay of MPB and PB which we published previously [10]. In extending the method to include many additional compounds, incorporation of a second derivatization was essential. PRM and its alkyl derivatives are extremely weak acids, and replacement of any protons on ring nitrogens requires an excess of strong base for catalysis. However the N-alkyl derivatives of PB, which are frequently present in the same samples, are reasonably labile to base-catalysed hydrolysis [9] and therefore undergo degradation. Fortunately, silylation with the relatively mild and somewhat selective reagent MTBSTFA enables analysis of some compounds which are not measurable following propylation. The two derivatizations permit at least one reliable determination to be made for each compound, and the extent of overlap provides valuable cross-checking for compounds which are adequately derivatized by both methods (e.g. **2a**, **2b** and **4b** in the example below). Fortunately a readily available internal standard (TPRM) was satisfactorily derivatized by both methods.

The capabilities and application of the procedure are illustrated for a compound of moderate metabolic complexity, N-methylprimidone (MPRM; **2b**). The probable metabolic routes of this substrate are C-2-oxidation (yielding **1b** and perhaps **6b**), N-demethylation (yielding **2a**) and/or aromatic hydroxylation (yielding **4b**). However, numerous secondary metabolic events are at least conceptually feasible. Thus **1a**, **4a** and **6a** could each be generated by N-demethylation of the precursors, **3b** could conceivably be formed from either **2a** or **4b** and could in turn give rise to **3a**, and both **5a** and **5b** are theoretically possible. Combinations of these simple, but well documented metabolic possibilities, therefore, suggest at least eleven possible metabolites from this substrate. This projection determines the mixture of standard compounds which would be considered appropriate for assay establishment and calibration for studies with this substrate.

Fig. 2 shows representative chromatograms for a typical set of standards, in this case those appropriate to MPRM as metabolic substrate. The same set of compounds are shown both alkylated and silylated, and several specific ion chromatograms are illustrated to exemplify the capabilities of the method. The compounds can be identified by mass number from the information provided in Table I. Thus the substrate, MPRM, has a protonated molecular ion at mass

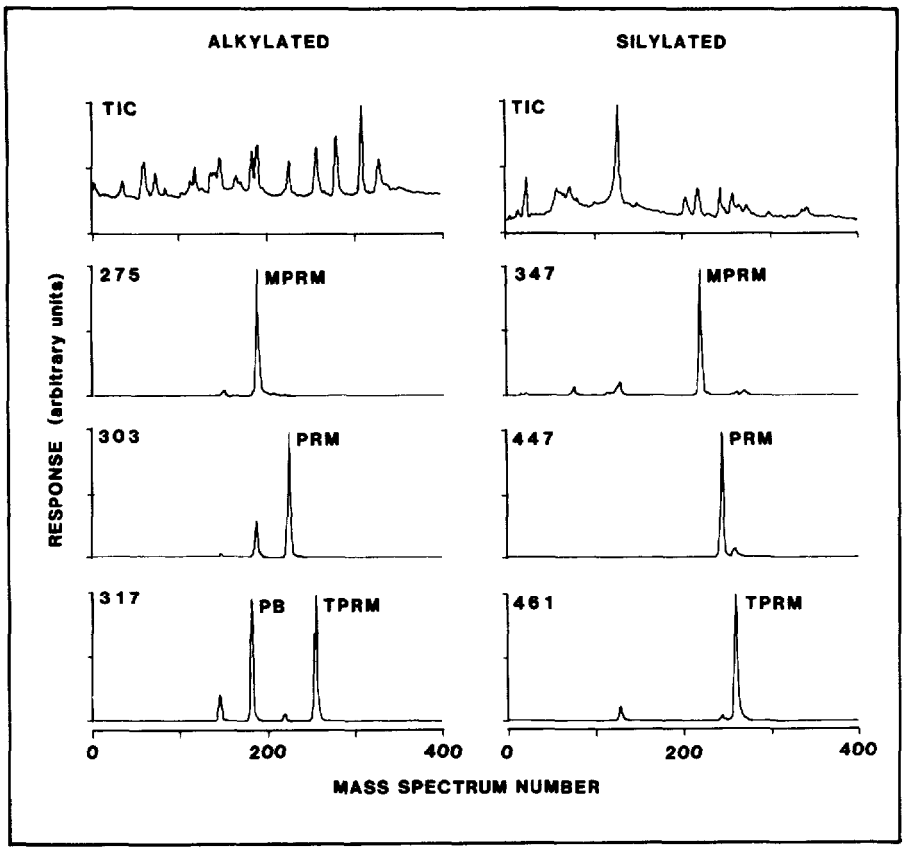

Fig. 2. Total ion chromatograms (TIC) and specific mass chromatograms for mixtures of metabolites of MPRM, following alkylation (left panel) and silylation (right panel). The compounds illustrated were derivatized by both procedures; other analytes were only measurable by one of the procedures (see Table I).

275 when mono-N-propylated and at mass 347 when mono-silylated. In some instances (e.g. masses 317 and 347 following alkylation), pairs of compounds give protonated molecular ions which are isobaric. However, in all instances where this occurred, the pairs were well resolved chromatographically.

Sets of standards containing mixtures of compounds appropriate to a substrate were processed, using ranges of concentrations predicted to exceed those encountered in urine. Clearly with some experience it became possible to refine these ranges. Linear calibration curves were obtained for each analyte in the presence of the others. The lowest concentration for the standards was typically 0.1 μg/ml, and this was generally assigned as the lower limit of quantification. The highest concentrations varied from 10 μg/ml for the alkyl barbiturates (which are not expected to be excreted unchanged in urine to any

TABLE I

GC–MS DATA FOR MPRM AND ITS POSSIBLE METABOLITES

Compound	Abbreviation	Alkylated		Silylated	
		MH^+	Retention time (min)	MH^+	Retention time (min)
2b	MPRM	275	13.53	347	15.60
2a	PRM	303	15.93	447	17.27
5b	MPEMA	–[a]	–	335	14.67
5a	PEMA	–	–	435	19.33
1b	MPB	289	10.67	–	–
1a	PB	317	13.13	–	–
4b	HOMPRM	333	18.67	477	20.87
4a	HOPRM	347	19.33	577	23.87
3b	MOMPB	347	19.53	–	–
3a	HOPB	375	21 40	–	–
6b	MPBOMC	377	22.67	–	–
6a	PBOMC	405	24.13	–	–
I.S.	TPRM	317	17.87	461	18.33

[a]Compound not derivatized satisfactorily by this procedure.

significant extent) to 200 μg/ml for the *p*-hydroxy compounds (which are predicted to be quantitatively important metabolites for most substrates). It should be noted that the hydroxy compounds are usually excreted in urine as conjugates with glucuronic acid, which is the major reason for including the β-glucuronidase hydrolysis step in the analytical method. It was always possible to obtain satisfactory linear calibration curves (i.e. r^2 values greater than 0.99) on the one day, but some variation (through a range of about 10%) in the slope of the curve was observed from day to day. This was not explained, but seemed to relate to the operating conditions of the mass spectrometer. Daily calibrations were therefore essential, and the excellent linearity of the procedure made it feasible to use a single concentration as daily calibrator; replicates of this were injected repeatedly throughout a day's batch of assays. The precision and accuracy of the method were within 10%, both within batches and from day to day.

The barbiturates and primidones which are asymmetrically substituted on the ring nitrogen atoms are chiral compounds. The present method does not involve enantioselective assay, though this refinement could potentially be incorporated by using a chiral capillary GC column. This may be a necessary addition for metabolic studies, since it has now been shown that the metabolism of MPB is characterized by extreme stereoselectivity [11], the *R*-enan-

tiomer having an apparent oral clearance some twenty-five to thirty times that of the *S*-enantiomer.

The method described in this paper has now been used for extensive metabolic studies of the twelve substrates; results of those studies will be presented in detail elsewhere. The method should be generally applicable to the determination of barbiturates and related compounds in complex mixtures. Representative chromatograms for the analysis of urine specimens (pre-dose and 12–24 h post-dose) from a volunteer who took N,N′-dimethylprimidone (**2d;** 300 mg orally) are shown in Fig. 3. This substrate has many potential metabolites in common with MPRM (**2b**), standards for which were discussed above. The major metabolites of this substrate and their cumulative recoveries over seven days, expressed as percentage of dose, were *p*-hydroxy-MPB (10.4%), N-methyl-PEMA (8.5%) and PEMA (2.7%).

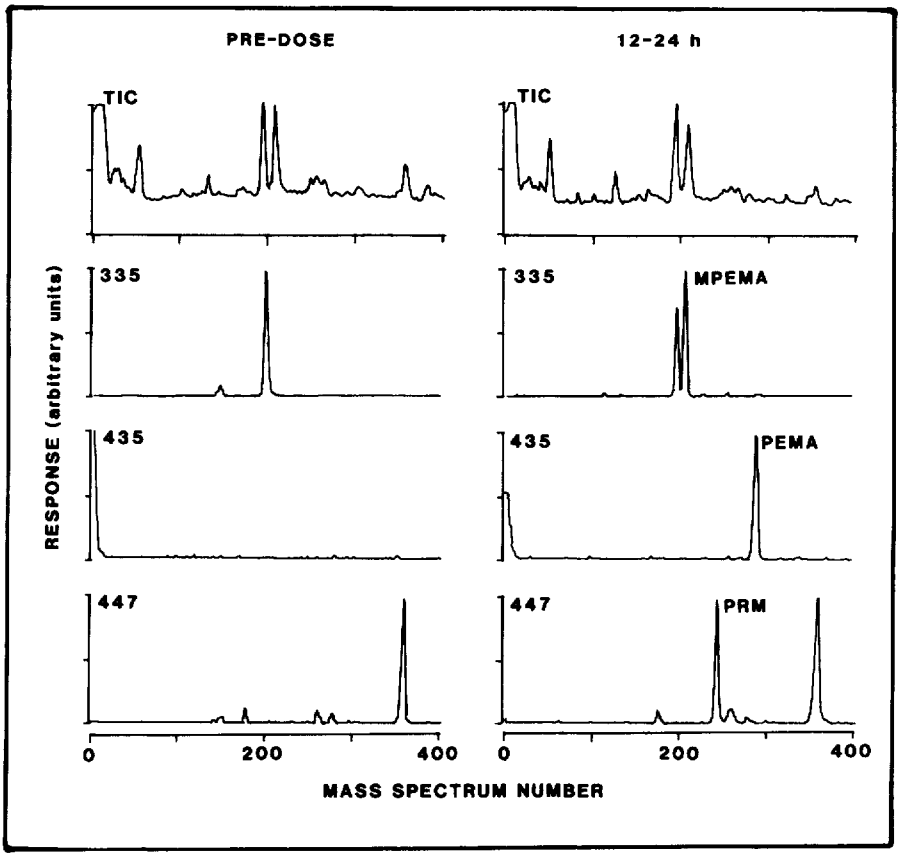

Fig. 3. Total ion chromatograms (TIC) and specific mass chromatograms for analysis, following silylation, of pre-dose (left panel) and 12–24 h post-dose (right panel) urine specimens from a volunteer who took N,N′-dimethyl-PRM. Responses in the left panel are not drug-related.

Clearly refinements such as the use of capillary columns and correspondingly higher mass spectral scan rates could enhance the method further. There is also scope for increasing sensitivity, if that were required, by using selected ion monitoring, as is easily possible with newer data systems. Nevertheless the method as presented has proven to be highly satisfactory for a complex and demanding analytical requirement.

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