Biomedical Mass Spectrometry 1976, 3, 60 to 63

A Method for the Estimation of Acetanilide, Paracetamol and Phenacetin in Plasma and Urine using Mass Fragmentography†

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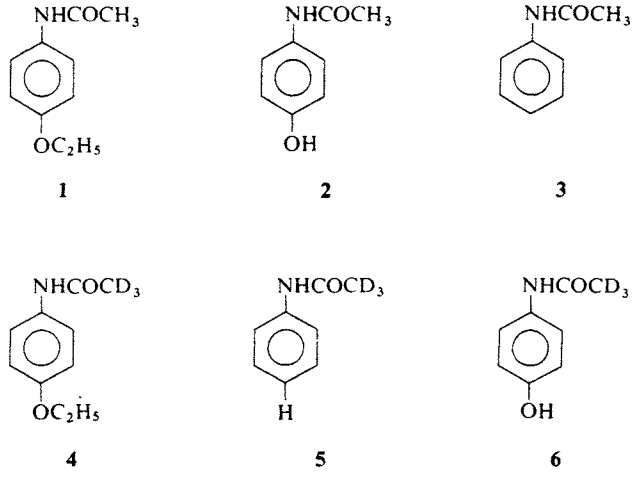
(*Received* 20 *October* 1975)

Abstract—Phenacetin, paracetamol and acetanilide can be determined in a plasma or urine sample by the use of deuterium labelled analogues. These are produced by reaction of hexadeuterioacetic anhydride with the appropriate aromatic amine. The $-NHCOCD_3$ group is stable to hydrogen exchange below pH 8. The internal standard is added to the plasma or urine after enzymatic hydrolysis of the paracetamol conjugates and an ethyl acetate extract at pH 5 is evaporated under nitrogen and the residue derivatized with *N,O*-bis-(trimethylsilyl)-acetamide. An aliquot of this solution is injected into a g.c.m.s. system, and one ion characteristic of the material under study and the ion from the deuterium analogue (3 mass units greater) are monitored using a voltage switching technique. In the case of phenacetin, for example, ions at 251 and 254 are monitored. Calibration curves relating different weight ratios of the hydrogen and deuterium compounds to their respective signals from the gas chromatograph mass spectrometer are used to calculate the amount of a compound in a particular sample. These methods have been developed to study the oxidation of acetanilide to paracetamol and the de-ethylation of phenacetin to paracetamol. Preliminary results from experiments with phenacetin will be discussed.

Introduction

PHENACETIN (**1**) and paracetamol (**2**) are analgesics in common use. These compounds and/or their metabolites may, however, be instrumental in producing liver and kidney damage.[1,2] Acetanilide (**3**), although it is no longer prescribed, can be useful as an index of aromatic hydroxylation.[3] The glucuronide conjugate of paracetamol is the principal metabolite of both phenacetin and acetanilide.[4] Gas chromatographic assays have been published for acetanilide,[5] phenacetin[6] and paracetamol.[7] These assays, although undoubtedly more specific and sensitive than the earlier spectrophotometric assays of Brodie,[8] are limited by the sensitivity of the flame ionization detector used and would not be expected to be viable at drug levels below 5 $\mu g\,ml^{-1}$.[7] A high speed liquid chromatographic assay for paracetamol has also been described.[9]

We are studying the metabolism of **1** and **3** in man after low dosage, and have developed simple and sensitive methods for the determination of these compounds using g.c.m.s. and mass fragmentography. The method enables all three compounds to be measured in a single plasma or urine sample. We have synthesized the deuterium labelled analogue of **1**, **2** or **3** by reaction of the appropriate aromatic amine and hexadeuterioacetic anhydride. These compounds are then used as internal standards in a mass fragmentographic assay, after conversion of the corresponding protio and deuterio compounds to their trimethylsilyl ethers. These compounds have excellent g.c.m.s. properties and have intense molecular ions or $(M - 15)^+$ ions.



Experimental

INSTRUMENTATION

Gas chromatographic mass spectrometric analyses were carried out using a Pye 104 gas chromatograph coupled via a silicone membrane separator to an A.E.I. MS-12 mass spectrometer operated at 8 kV, with a filament current of 300 μA and an ionizing voltage of 25 eV. The analogue data were analysed by a PDP 8/I computer (Digital Equipment Corp, Maynard, Massachusetts) with a 4K memory and a 256K disc store. A commerical data system (A.E.I. DS-30) was modified

† Abbreviations: BSA = *N,O*-bis-(trimethylsilyl)acetamide; Regisil = bis-(trimethylsilyl)trifluoroacetamide; TRI-SIL Z = trimethylchlorosilane + BSA + trimethylsilylimidazole.

for this work. The g.c. system used 5 ft × $\frac{1}{8}$ in glass columns packed with 3% OV17 on 100/120 mesh Gas Chrom. Q.

Mass fragmentography was done using a six channel multi-peak monitor unit manufactured by A.E.I. This system is similar to that developed by Hammar.[10] The signal from each selected mass value was displayed on a Rikadenki multi-channel chart recorder.

MATERIALS

All solvents were of 'Analar' standard and were distilled twice before use. Hexadeuterioacetic anhydride (99%) and trideuterioacetic acid (98%) were obtained from the Aldrich Chemical Co. β-Glucuronidase (Type H2) was obtained from the Sigma Chemical Company. The activity of 1 ml of this preparation was 134 000 Fishman units. Trimethylsilyl ethers were prepared using *N,O*-bis-(trimethylsilyl)acetamide (BSA), supplied by B.D.H. Chemicals, Poole, Dorset. Regisil [bis-(trimethylsilyl)trifluoroacetamide] was obtained from Field Instruments, London. TRI-SIL Z [trimethylchlorosilane + BSA + trimethylsilylimidazole ((2:3:3)] was obtained from Phase Separations Ltd, Connah's Quay, Flintshire.

The deuterium labelled compounds **4** and **5** used in this work were synthesized by heating the appropriate aromatic amine with an equimolar amount of hexadeuterioacetic anhydride. Typically, 0.5 ml of aniline or *p*-phenetidine was heated for 1 h at 70 °C with 0.6 ml of hexadeuterioacetic anhydride. On cooling in ice, the appropriate amide precipitated from solution. After filtration, the ^{2}H-acetanilide was recrystallized from water and ^{2}H-phenacetin was recrystallized from absolute alcohol. The overall yield was 70%. ^{2}H-Acetanilide melted at 110–112 °C and ^{2}H-phenacetin melted at 130–132 °C. A modification of the above procedure was used for the synthesis of ^{2}H-paracetamol (**6**). It was found that direct acetylation of *p*-aminophenol with an equimolar amount of anhydride produced a mixture of the mono- and diacetyl compounds. The desired monoacetyl compound was produced by the reaction of *p*-aminophenol with an equimolar amount of the anhydride and a fourfold molar excess of trideuterioacetic acid. After recrystallization from water the ^{2}H-paracetamol melted at 166 °C.

Trimethylsilyl ethers of the protio and deuterio compounds were prepared by heating at 80 °C for 30 min with BSA. Regisil was also effective in producing a di-TMS-ether from paracetamol but TRI-SIL Z produced only a mono-TMS-ether. The mass spectra of the TMS derivatives of compounds **4**, **5** and **6** are shown in Figs. 1, 2 and 3. An intense molecular ion is found in every case associated with an intense ion at $[M - 15]^+$. Subsequent fragmentation involves loss of the sidechain. Careful comparison was made between the mass spectra of the ^{2}H and ^{1}H forms of each TMS derivative. No evidence of isotopic rearrangement was found.

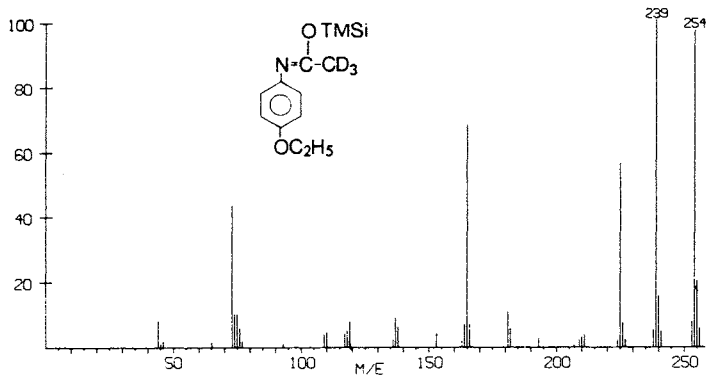


FIG. 1. The mass spectrum of TMS ^{2}H-phenacetin.

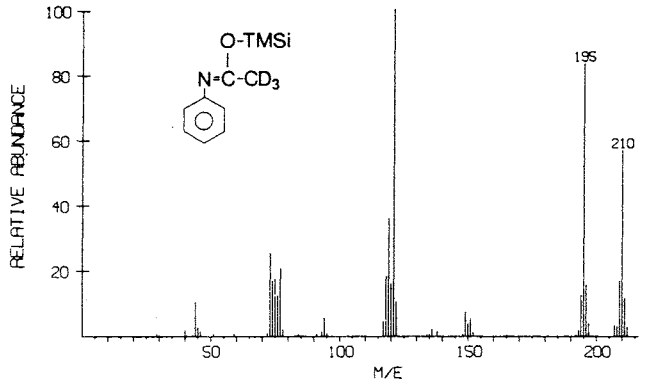


FIG. 2. The mass spectrum of TMS ^{2}H-acetanilide.

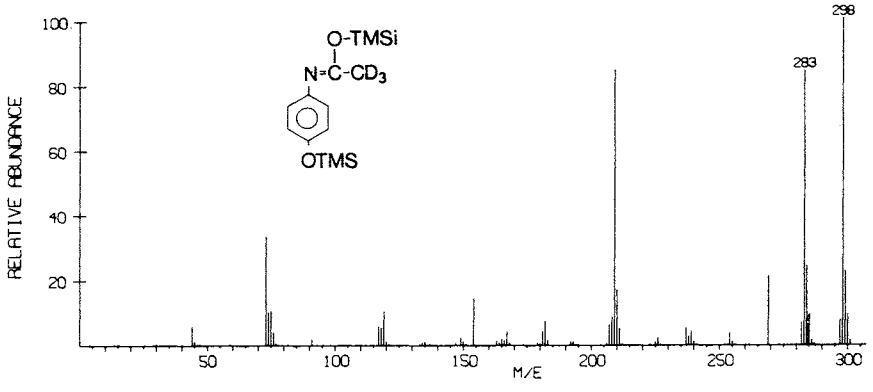


FIG. 3. The mass spectrum of di-TMS ^{2}H-paracetamol.

STABILITY OF THE DEUTERIO COMPOUNDS

To be viable as internal standards the ^2H deuterium atoms in compounds **4**, **5** and **6** should not exchange under the conditions of extraction, derivatization and g.c.m.s. analysis. The mass spectral data confirm the stability of the label during derivatization and analysis. To determine the stability of the label during the extraction procedure, 1 ml of an aqueous solution containing 1 mg of the ^2H labelled compound was vortexed for 2 min with 5 ml of a buffer solution and allowed to stand for 1 h. Phthalate buffer (0.05 M) at pH 4, 6, 7 and 10 was used. After extraction from aqueous solution each compound was then subjected to g.c.m.s. analysis and the intensities of peaks in the molecular ion region compared with those of the pure ^2H and ^1H compounds. No difference in the pattern or intensity of the ions around the molecular ion region was found for any of the labelled compounds treated with the buffer solution. However, on repeating the experiment with a 10% solution of sodium hydroxide, deuterium exchange was apparent. All extractions of authentic samples were thus carried out at an acid pH.

CONSTRUCTION OF STANDARD LINES

Quantitation of compounds **1**, **2** and **3** was based on the peak height ratio of a suitable *m/e* value for ^1H material to the *m/e* value three mass units higher, corresponding to a known amount of labelled internal standard.

Very good correlation could be obtained between peak heights of ^2H- and ^1H-phenacetin without derivatization. Paracetamol and acetanilide, however, gave unsymmetrical g.c. peaks and poor g.c.m.s. linearity at the nanogram level. Using the TMS derivatives excellent g.c. and g.c.m.s. response was obtained, and the increase in mass provided by the addition of one or more TMS groups ensured a higher degree of specificity and separation from background contamination.

The *m/e* values chosen for this assay were the molecular ions of TMS ^1H-phenacetin and ^2H-phenacetin at *m/e* 251 and 254, and the molecular ions of TMS ^1H-paracetamol and ^2H-paracetamol at *m/e* 295 and 298. The molecular ions of the acetanilide TMS derivatives (*m/e* 207 and 210) were not used because of the large background signal at *m/e* 207 due to silicone bleed from the molecular separator and the g.c. phase. The $[M - 15]^+$ ions of ^1H-acetanilide were found to be satisfactory for the analysis. Samples consisting of 1 ml blank plasma were spiked with 100, 200, 500 and 1000 ng of **1**, **2** and **3**, and analysed by the method outlined below. The calibration graph for phenacetin against the deuterio analogue is shown in Fig. 4. For the solutions containing 1% by weight of the proteo compound the amount of material injected into the g.c.m.s. system was 4 ng. The data from these curves were analysed by a least-squares regression line. The 95% confidence limits (standard deviation times Student's t ($n = 16$)) for duplicate analysis were

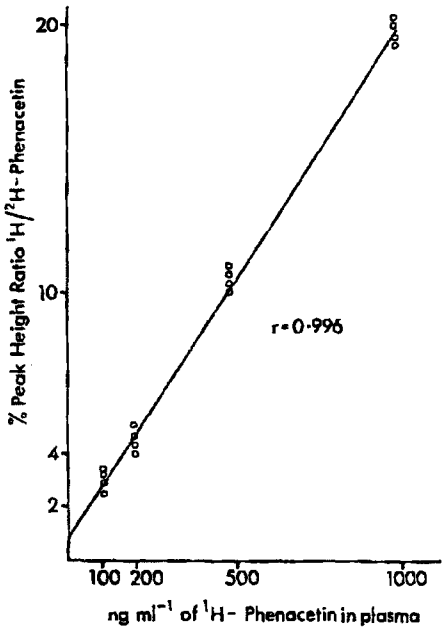


FIG. 4. Calibration of ^1H-phenacetin and ^2H-phenacetin from plasma. The actual amounts of material injected into the gas chromatograph mass spectrometer were 1/25th of the total added to plasma.

± 31 ng ml^{-1}, ± 32 ng ml^{-1} and ± 35 ng ml^{-1} for paracetamol, phenacetin and acetanilide, respectively.

EXTRACTION AND ANALYSIS

The labelled internal standard (2–10 μg) was added to 1 ml of plasma. The mixture was then buffered to pH 5.0 with 0.4 ml of 3 M NaH_2PO_4 and extracted with ethyl acetate (10 ml). After centrifugation at 2500 r.p.m. for 15 min the organic layer was transferred to a conical centrifuge tube and evaporated to dryness under a stream of nitrogen. The residue was reacted with 50 μl of BSA as described above and 2 μl of this solution was injected into the gas chromatograph mass spectrometer. To determine the total amount of paracetamol present the plasma was hydrolysed by adding 100 μl of β-glucuronidase at pH 5.0 and incubating for 18 h at 37 °C. The sample was then processed as described above. Samples of blank plasma were extracted and converted to TMS derivatives. They gave no response at the mass values used in the analysis. Recovery of compounds added to plasma and extracted by this method was greater than 70%. Although this is not as high as might be expected the use of an internal standard of nearly identical chemical structure compensates for losses involved in extraction.

Results and discussion

Figure 5 shows the time course of phenacetin metabolism in five volunteers who ingested approximately 100 mg of phenacetin (actual dose was related to metabolically active mass = body wt kg$^{0.7}$). Using the method described above we were able to measure plasma levels of phenacetin down to 20 ng ml^{-1}. The known inter-subject variation in phenacetin levels is

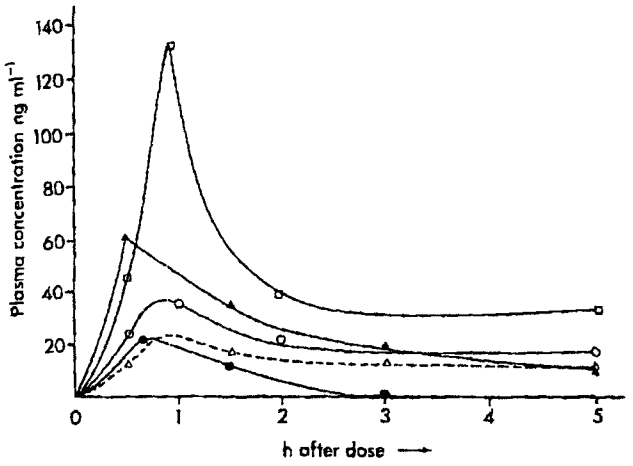


FIG. 5. Plasma levels of phenacetin in five volunteers following an oral dose of 100 mg of phenacetin.

apparent.[11] The paracetamol levels in these studies are of the order 1–2 μg ml^{-1}. The pharmacokinetic data from this study will be published elsewhere. One problem encountered in this method is the deterioration in g.c.m.s. performance after several samples have been analysed. This is caused by high molecular weight compounds, such as the TMS derivative of cholesterol, gradually leaving the g.c. column, entering the ion source and producing a high background signal. We have recently overcome this problem by using a t.l.c. 'clean-up' procedure which we have shown to be successful for paracetamol assays, and which we are currently developing for acetanilide and phenacetin. The initial plasma or urine extract is applied to a t.l.c. plate of silical gel (250 μm, Merck) and developed in a solvent system consisting of benzene + methanol + acetic acid (45:8:4). The t.l.c. spot corresponding to the ^{2}H- and ^{1}H-paracetamol is removed and derivatized by direct reaction with BSA. The supernatant BSA solution is then injected directly into the gas chromatograph mass spectrometer. Recovery of the amide and its internal standard from the plate is greater than 80% and no exchange occurs in the acidic developing system.

The g.c.m.s. system is able to distinguish the ions associated with each compound. The g.c.m.s. behaviour of TMS acetanilide is such that the analysis of the compound is best performed at a temperature of 100 °C. The g.c. retention time of TMS acetanilide at this temperature is approximately 2 min with a g.c. flow rate of 30 ml min^{-1}. Phenacetin and paracetamol are eluted at a temperature of 160 °C (retention times 3 min) as two closely spaced peaks. It is quite feasible to use ^{2}H-phenacetin as the internal standard for the estimation of paracetamol. The plot of differing weight ratios of ^{2}H-phenacetin/^{1}H-paracetamol against *m/e* 254/280 has a correlation coefficient of 0.9987. Programmed switching of selected groups of *m/e* values corresponding to an unknown and its internal standard should enable multi-component mixtures to be analysed in one g.c.m.s. profile.

ACKNOWLEDGEMENTS

We thank Mrs P. A. Robinson for valuable technical assistance. We also thank the Nuffield Foundation for financial support, and the M.R.C. for provision of a Studentship (P.R.). We thank Professor D. A. Price Evans for advice and encouragement during the course of this work.

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