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An Assay for Paracetamol, Produced by the *O*-Deethylation of Phenacetin *in vitro*, Using Gas Chromatography/Electron Capture Negative Ion Chemical Ionization Mass Spectrometry

S. Murray† and A. R. Boobis
Department of Clinical Pharmacology, Royal Postgraduate Medical School, Ducane Road, London W12 0HS, UK

A sensitive gas chromatographic/mass spectrometric assay for paracetamol, produced by the *O*-deethylation of phenacetin by microsomal fractions of human liver, has been developed. The method uses the 3,5-bistrifluoromethylbenzoyl derivative of paracetamol and electron capture negative ion chemical ionization. This derivative has a negative ion mass spectrum which contains only a molecular ion at *m/z* 391 and, when this ion is specifically monitored, an amount of derivative equivalent to 1 pg of paracetamol can be detected. ($^2H_3$)Paracetamol was available for use as an internal standard and this allowed the development of an assay with a precision of 8% (SD) at 0.5 ng paracetamol per microsomal incubation and a lower limit of quantitative determination of 0.2 ng paracetamol per microsomal incubation.

INTRODUCTION

Paracetamol (4-hydroxyacetanilide) is produced by the *O*-deethylation of phenacetin both *in vivo* and *in vitro* by a hydrocarbon-inducible reaction. As part of our studies on the multiplicity of hepatic cytochromes P-450, we wished to develop an assay which would enable this *O*-deethylation reaction to be studied in microsomal fractions of human liver. The assay, as well as being specific, had to be sensitive, since the amount of human liver available was limited. Because paracetamol itself is very widely used as an analgesic, many methods have been developed for measuring the compound in biological fluids. However, while the methods based on gas chromatography[1-3] and high-performance liquid chromatography[4-6] are adequate for monitoring levels of the drug in blood following a therapeutic dose, they are not sufficiently sensitive for our needs.

Combined gas chromatography/mass spectrometry (GC/MS) can provide greater sensitivity than the above methods and two assays for paracetamol in plasma using GC/MS and selected ion monitoring (SIM) have been described. Garland *et al.*[7] employed the methyl derivative of paracetamol and positive ion chemical ionization with isobutane as reagent gas, quoting a detection limit of 100 ng ml^{-1}. Baty *et al.*[8] used the ditrimethylsilyl derivative of paracetamol and electron impact ionization but did not give a lower limit of quantification. A method for measuring paracetamol produced in microsomal incubations based on GC/MS and SIM has also been published.[9] This uses the ditrimethylsilyl derivative of paracetamol and electron impact ionization and has a limit of quantitation of 5 ng paracetamol per incubation.[10] Recently, work on drug analysis by GC/MS has been published describing the use of bistrifluoromethylaryl derivatives coupled with electron capture negative ion chemical ionization (CI).[11-14] Under these conditions, such derivatives give an intense negative ion current. The mass spectra contain prominent high-mass ions and SIM of these allows detection of one picogram or less of the parent compound.[11-14] Consequently we synthesized the 3,5-bistrifluoromethylbenzoyl derivative of paracetamol to determine whether this compound would provide the basis for an assay with the required increase in sensitivity.

EXPERIMENTAL

Chemicals

Phenacetin and paracetamol were obtained from BDH Chemicals Ltd (Poole, UK). Phenacetin was purified prior to use by dissolving in chloroform and washing with aqueous sodium hydroxide solution (this removed any trace amounts of paracetamol which were present). ($^2H_3$)Paracetamol was synthesized by treating *p*-aminophenol with a mixture of hexadeuteroacetic anhydride and trideuteroacetic acid.[8] NADPH (Type 1), diisopropylethylamine and undecane were purchased from Sigma Chemical Co. Ltd (Poole, UK) while 3,5-bistrifluoromethylbenzoyl chloride was supplied by Fluorochem Ltd (Glossop, UK). Solvents and other chemicals were all of analar grade. Before use, ethyl acetate was distilled from and stored over calcium hydride.

Microsomal incubations

Each incubation contained 75 mM tris-HCl buffer (pH 7.4), 3 mM magnesium chloride, 1.2 mM NADPH, 10-50 μg microsomal protein and phenacetin in a final volume of 1 ml. The reaction was started by addition of the phenacetin at 50 fold the required final concentration in 20 μl methanol. Incubation was for 10 min at 37 °C

† Author to whom correspondence should be addressed.

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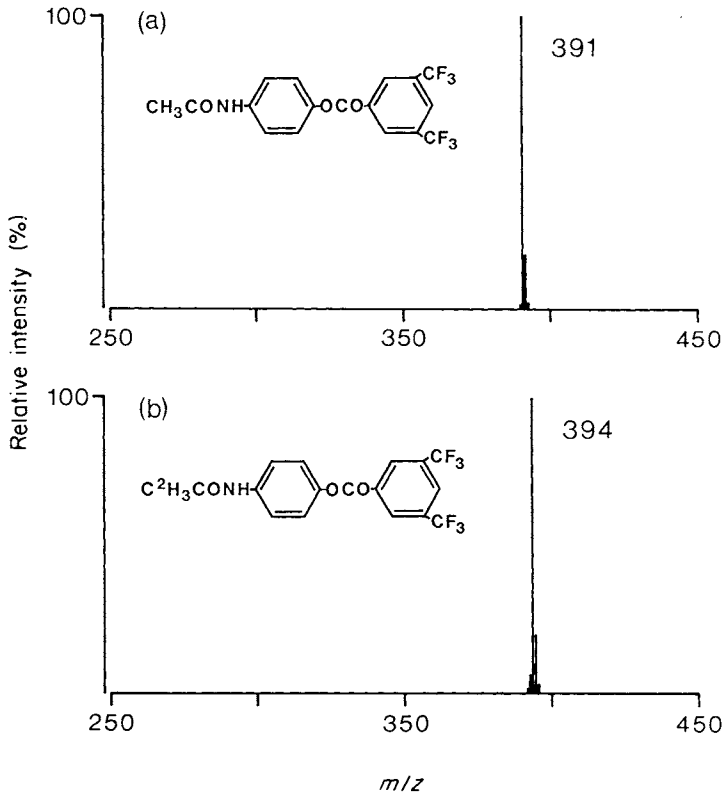

Figure 1. The electron capture negative ion CI mass spectra of the 3,5-bistrifluoromethylbenzoyl derivatives of (a) paracetamol and (b) ($^2H_3$)paracetamol.

in air in a shaking water bath and the reaction was terminated by addition of 300 μl of ice-cold 0.5M sodium hydroxide solution (taking the pH to 12) and removal to an ice bath. Standards were prepared by adding known amounts of paracetamol in methanol to incubation samples in which the phenacetin was excluded until after the sodium hydroxide had been added.

Extraction procedure

To each alkaline sample was added ($^2H_3$)paracetamol (10 ng in 50 μl methanol). Diethyl ether (15 ml) was added to each tube and, after vortex mixing and a brief centrifugation, the organic layer was removed by aspiration and discarded. To the aqueous phase remaining was added 1 M potassium phosphate buffer (pH 7.0, 1 ml) which took the pH to 7.1 (this neutralization should be carried out within an hour of the addition of sodium hydroxide). Diethyl ether (15 ml) was added and, after vortex mixing followed by centrifugation, the upper organic layer was transferred to a clean tube and evaporated to dryness under nitrogen. The residue was dissolved in two 0.5 ml aliquots of ether which were transferred to a 1 dram glass vial. The ether was evaporated to dryness once more and methanol (100 μl) added. Samples were then stored at 4 °C prior to derivatization.

Derivatization procedure

Methanol added to samples prior to storage was removed by evaporation under nitrogen. To each vial was added dry ethyl acetate (40 μl) containing 3,5-bistrifluoromethylbenzoyl chloride (0.4 μl) and diisopropylethylamine (0.4 μl). The reaction mixture was left at room temperature for 30 min and then evaporated to dryness under nitrogen. The residue was reconstituted in 50 μl undecane and 2 μl aliquots were injected into the gas chromatograph/mass spectrometer.

Gas chromatography/mass spectrometry

A Finnigan-MAT 4500 combined gas chromatograph/quadrupole mass spectrometer system (Finnigan-MAT, San Jose, California 95134, USA) was used. The gas chromatograph was equipped with a 30 m SE54 J & W fused silica capillary column which was routed through the separator oven (maintained at 250 °C) and directly into the mass spectrometer ion source. Helium was used as carrier gas at a head pressure of 20 psi. The gas chromatograph was fitted with a Grob-type capillary injector operated in the splitless mode and maintained at a temperature of 250 °C. The gas chromatograph oven temperature was held at 160 °C for 1 min, then raised to 280 °C at 20 °C min^{-1}. Under these conditions, the retention times of the bistrifluoromethylbenzoyl derivatives of paracetamol and ($^2H_3$)paracetamol were 5.4 min. The mass spectrometer was operated in the negative ion CI mode with an electron energy of 100 eV. Ammonia gas was admitted via a Negretti and Zambra needle valve to an indicated ion source pressure of 0.35 Torr and the indicated ion source temperature was maintained at 150 °C. The mass spectrometer was tuned to monitor negative ions at m/z 391 and m/z 394 and data

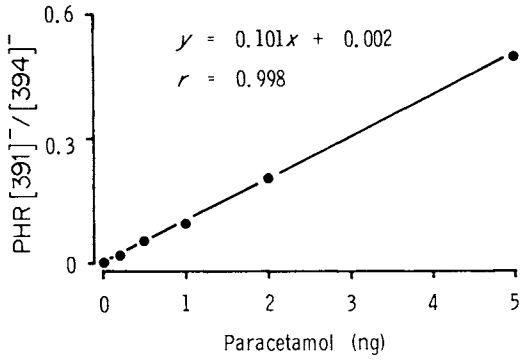

Figure 2. Standard curve for paracetamol extracted from microsomal incubations.

acquisition and reduction were performed by an INCOS data system using IDOS 1 software.

RESULTS AND DISCUSSION

The electron capture negative ion Cl mass spectrum of the 3,5-bistrifluoromethylbenzoyl derivative of paracetamol is shown in Fig. 1(a). The base peak in the mass spectrum is the molecular ion at m/z 391 and there are no significant fragment ions. When negative ions with m/z 391 are specifically monitored, an amount of product equivalent to 1 pg of paracetamol can be detected with a signal-to-noise ratio of 40:1. This derivative therefore has the potential of providing a very sensitive gas chromatographic/mass spectrometric assay for paracetamol. ($^2H_3$)Paracetamol is available for use as an internal standard and the negative ion mass spectrum of the 3,5-bistrifluoromethylbenzoyl derivative of this compound is shown in Fig. 1(b). The mass spectrum is analogous to that of the unlabelled compound and contains a single major ion at m/z 394. Ions with m/z 391 are less than 1% of the abundance of the base peak.

A standard curve for paracetamol extracted from microsomal incubations was prepared. Blank incubations were spiked with 10 ng of ($^2H_3$)paracetamol and up to 5 ng of paracetamol. Paracetamol has a pK_a of 9.8 and so, by altering the pH to 12 with sodium hydroxide, 96% of unmetabolized phenacetin can be selectively removed by extraction with ether.[10] Paracetamol is stable at pH 12 for at least one hour[10] while ($^2H_3$)paracetamol does not show any deuterium exchange during this period.[10] The incubation, extraction and derivatization procedures are described in detail in the experimental section. The mass spectrometer was tuned to monitor negative ions at m/z 391 and m/z 394. There was no interference from other compounds in either ion channel and, over the range of 0–5 ng, the standard curve was linear with an intercept close to zero (Fig. 2). The precision for measurement of paracetamol in microsomal incubations is 8% (SD, $n=6$) at 0.5 ng and the lower limit for quantitative determination is 0.2 ng.

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