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Combined assay for phenacetin and paracetamol in plasma using capillary column gas chromatography–negative-ion mass spectrometry

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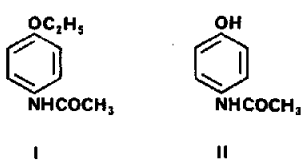
ABSTRACT

A gas chromatographic–mass spectrometric assay has been developed for the measurement of phenacetin and its major metabolite paracetamol in plasma. Phenacetin and unconjugated paracetamol are analysed in a single chromatographic run while total paracetamol is measured separately after enzymatic hydrolysis. The two compounds, and the deuterated analogues used as internal standards, are analysed as their trifluoroacetyl derivatives and the mass spectrometer is operated in the electron-capture negative-ion chemical ionisation mode. The negative-ion mass spectra of the derivatives contain fragment ions, formed by loss of an acetyl group from the respective molecular ions, which are the base peaks in the spectra. When these ions are specifically monitored, amounts of derivative equivalent to 1 pg of parent compound can be detected. This allowed the development of an assay for phenacetin, unconjugated paracetamol and total paracetamol in plasma having a precision of 2.6, 1.4 and 2.4%, respectively, and preliminary results for a subject given a 100-mg oral dose of phenacetin are reported.

INTRODUCTION

Phenacetin (I, Fig. 1), at one time a widely consumed medication because of its analgesic and antipyretic properties, is no longer prescribed because of the risks of renal damage [1] and methemoglobinaemia [2] associated with its long-term use. However, it is still of considerable interest as a specific substrate for one isoenzyme of cytochrome P450 in man, P450IA2, which is responsible for producing the major metabolite paracetamol (II, Fig. 1) [3]. Phenacetin therefore has the potential to serve as a probe of P450IA2 activity *in vivo* and, as part of our



I II

Fig. 1. Chemical structures of phenacetin (I) and paracetamol (II).

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work in this area, we required a method for measuring phenacetin and paracetamol in human plasma.

Assays for these two compounds developed previously by other workers involved high-performance liquid chromatography (HPLC) [4–6] or gas chromatography–mass spectrometry (GC–MS) with selected-ion monitoring (SIM) of positive ions [7,8]. However, because we wished to administer low doses of phenacetin (to minimise the risk of possible adverse effects) and because the compound is subject to extensive first-pass metabolism, blood levels of the parent drug would be low and so a very sensitive assay for phenacetin was required. Also, to facilitate population studies involving a large number of samples, a method where phenacetin and paracetamol could be measured simultaneously was desirable. We have therefore developed, and describe here, an assay for phenacetin and unconjugated and total paracetamol in plasma, based on GC–MS with SIM of negative ions and using a derivative common to both compounds.

EXPERIMENTAL

Chemicals

Phenacetin and paracetamol were obtained from BDH (Poole, UK) and N-(4-hydroxyphenyl-2,3,5,6-d_4)acetamide ([$^2H_4$]paracetamol) from MSD Isotopes (Montreal, Canada). 4-Aminophenol, pentadeuteroethyl iodide, hexadeuteroacetic anhydride and tetradeuteroacetic acid were supplied by Aldrich (Gillingham, UK). Type H1 sulphatase and octane were purchased from Sigma (Poole, UK) while trifluoroacetic anhydride was purchased from Pierce and Warriner (Chester, UK). Organic solvents were all of Analar grade and dry ethyl acetate was generated by distillation from and storage over calcium hydride.

Synthesis of deuterated standards

N-(4-Pentadeuteroethoxyphenyl)acetamide ([$^2H_5$]phenacetin). A mixture of N-(4-hydroxyphenyl)acetamide (paracetamol, 4.5 g, 30 mmol), potassium carbonate (6.9 g, 50 mmol), pentadeuteroethyl iodide (5 g, 31 mmol) and acetone (40 ml) was refluxed for 23 h. After cooling, chloroform (100 ml) and water (50 ml) were added and the resulting mixture was transferred to a separating funnel. The organic layer was isolated, washed with 1 *M* sodium hydroxide solution (2 × 50 ml), water (50 ml) and then dried over magnesium sulphate. After filtration and removal of the solvent, a crystalline white residue remained which was washed with hexane. Yield 4.2 g (76%); m.p. 131°C.

N-(4-Hydroxyphenyl)trideuteroacetamide ([$^2H_3$]paracetamol). This compound was synthesised by reacting 4-aminophenol with hexadeuteroacetic anhydride, in the presence of tetradeuteroacetic acid, as described by Baty *et al.* [7]; m.p. 164°C (lit. [7] m.p. 166°C).

Preparation of samples for extraction

Measurement of phenacetin and unconjugated paracetamol. To plasma (500 μl) in a 10-ml screw-capped glass tube were added deionised water (500 μl), [$^2H_5$]-phenacetin (20 ng in 100 μl methanol) and [$^2H_4$]paracetamol (1 μg in 100 μl methanol). Tube contents were then mixed by vortex agitation.

Measurement of total paracetamol. To plasma (100 μl) in a 10-ml screw-capped glass tube were added deionised water (400 μl) and a solution of type H1 sulphatase (100 U), also containing β-glucuronidase activity (1200 U), in 0.2 *M* acetate buffer (500 μl) at pH 5.5. The tube was capped, shaken gently to ensure mixing of contents, then placed in a heated water-bath at 37°C for 16 h. After removal from the waterbath, [$^2H_4$]paracetamol (200 ng in 200 μl methanol) was added and the tube contents mixed by vortex agitation.

Extraction procedure

Diethyl ether (8 ml) was added to each sample, the tube securely capped and the contents thoroughly mixed by manual inversion. After centrifugation (10 min at 750 *g*), the upper organic layer was transferred to a clean glass tube and evaporated to dryness under a gentle stream of nitrogen. The residue was transferred to a glass vial (2 ml capacity) with methanol (2 × 750 μl) and stored at −20°C until required for derivatisation and analysis by GC–MS.

Derivatisation procedure

Methanol was removed from sample extracts by evaporation under nitrogen. To the dried residue in each vial was added dry ethyl acetate (100 μl) containing trifluoroacetic anhydride (10 μl), the vial then being capped and left to stand for a minimum period of 30 min at room temperature. Just prior to analysis by GC–MS, derivatising reagent was removed by evaporation under nitrogen and the residue was reconstituted in octane (20 μl for phenacetin and unconjugated paracetamol analysis, 100 μl for total paracetamol analysis). Aliquots of 2 μl were injected into the gas chromatograph–mass spectrometer.

Gas chromatography–mass spectrometry

A Finnigan MAT 4500 combined gas chromatograph–quadrupole mass spectrometer (Finnigan MAT, San Jose, CA, USA) was used. The gas chromatograph was equipped with a 15 m × 0.25 mm I.D. DB1701 J&W fused-silica capillary column (Folsom, CA, USA) which was routed through the separator oven (maintained at 290°C) and directly into the mass spectrometer ion source. Helium was used as carrier gas at a head pressure of 69 kPa. The gas chromatograph was fitted with a Grob-type capillary injector operated in the splitless mode and maintained at a temperature of 270°C. The gas chromatograph oven temperature was held at 110°C for 1 min, then raised to 210°C at 20°C/min. Under these conditions, the retention times of the trifluoroacetyl derivatives of phenacetin and paracetamol were 4.25 and 3.45 min, respectively. The mass spec-

trometer was operated in the negative-ion chemical ionisation (NICI) mode with an electron energy of 100 eV. Ammonia gas was admitted to an indicated ion source pressure of 53 Pa and the indicated ion source temperature was maintained at 150°C. The mass spectrometer was tuned to monitor negative ions at *m/z* 232, 237, 343 and 347 when analysing for phenacetin and unconjugated paracetamol, and negative ions at *m/z* 246 and 250, or *m/z* 300 and 304, when analysing for total paracetamol. Data acquisition and reduction were performed by an INCOS data system using IDOS 2 software.

Standard curves

Measurement of phenacetin and unconjugated paracetamol. Six standard methanol solutions in glass vials (2 ml capacity) were prepared from stock solutions of phenacetin (100 ng/ml in methanol), [$^2H_5$]phenacetin (200 ng/ml in methanol), paracetamol (10 μg/ml in methanol) and [$^2H_4$]paracetamol (10 μg/ml in methanol). The standards all contained 20 ng [$^2H_5$]phenacetin and 1 μg [$^2H_4$]paracetamol, as well as amounts of phenacetin in the range 0–50 ng and paracetamol in the range 0–1.5 μg. After evaporation to dryness under nitrogen, standards were taken through the derivatisation procedure described above.

Measurement of total paracetamol. Six standard methanol solutions in glass vials (2 ml capacity) were prepared from stock solutions of paracetamol (1 μg/ml in methanol) and [$^2H_4$]paracetamol (1 μg/ml in methanol). The standards all contained 200 ng [$^2H_4$]paracetamol as well as amounts of paracetamol in the range 0–300 ng. After evaporation to dryness under nitrogen, standards were taken through the derivatisation procedure described above.

RESULTS AND DISCUSSION

The major requirements of an assay based on GC–MS are that the compound of interest shows good chromatographic behaviour and has a mass spectrum containing prominent, high-mass ions suitable for SIM. Good chromatography can usually be achieved by derivatisation of polar groups in the molecule and, if a fluorinated reagent is used for this procedure, the halogenated reaction product normally gives an intense ion current under conditions of electron-capture (EC) NICI-MS. We have successfully used the latter procedure as the basis for the analysis of several exogenous [9–12] and endogenous [13,14] compounds and a similar approach was used here for the measurement of phenacetin and paracetamol.

A combined assay for phenacetin and paracetamol requires the use of a common derivative and hence a reagent capable of reacting with the amide group present in both compounds. Trifluoroacetic anhydride reacts with phenacetin at room temperature to give a mono-trifluoroacetyl (TFA) derivative [15] and with paracetamol to give a di-TFA derivative, and the ECNICI mass spectra of the two compounds are shown in Fig. 2. Both spectra contain small molecular ions

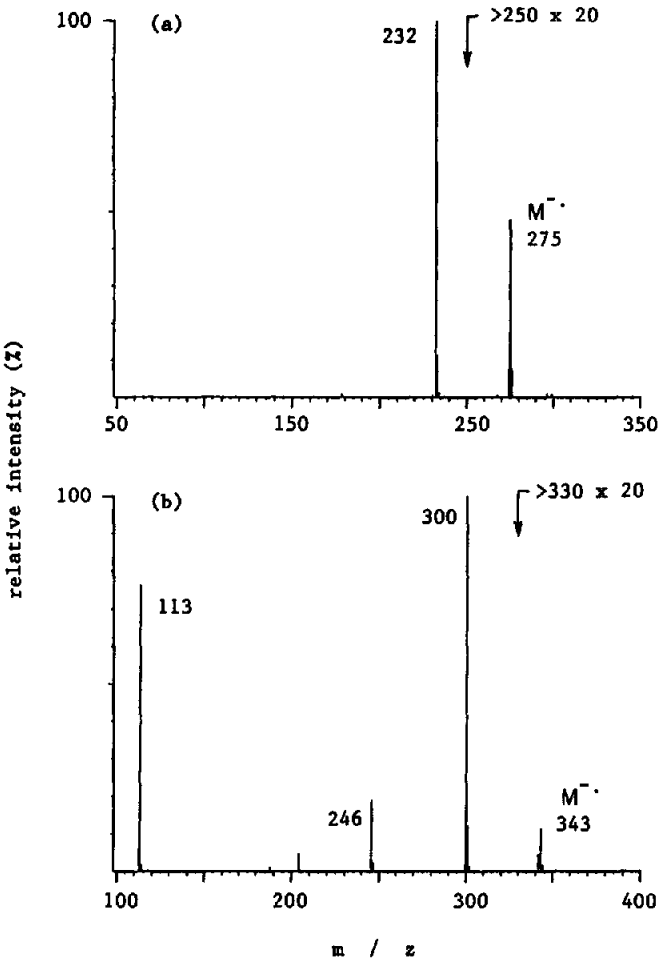

Fig. 2. ECNICI mass spectra of the TFA derivatives of (a) phenacetin and (b) paracetamol.

(phenacetin, *m/z* 275; paracetamol, *m/z* 343), with the base peaks (phenacetin, *m/z* 232; paracetamol, *m/z* 300) corresponding to loss of an acetyl group from the respective molecular ions. The derivatives had good GC properties with no sign of peak broadening or tailing, and, when the base peaks were specifically monitored, amounts of derivative equivalent to 1 pg of phenacetin (signal-to-noise ratio >20) and paracetamol (signal-to-noise ratio ~ 100) could be detected.

TFA derivatives of phenacetin and other amides have been reported to be rather unstable [15]. We also found that, with the removal of the derivatising reagent, after half an hour the response of the TFA derivatives of phenacetin and paracetamol on GC–MS began to decline. Consequently, it was decided that the derivatising reagent should be removed, by evaporation under nitrogen, only

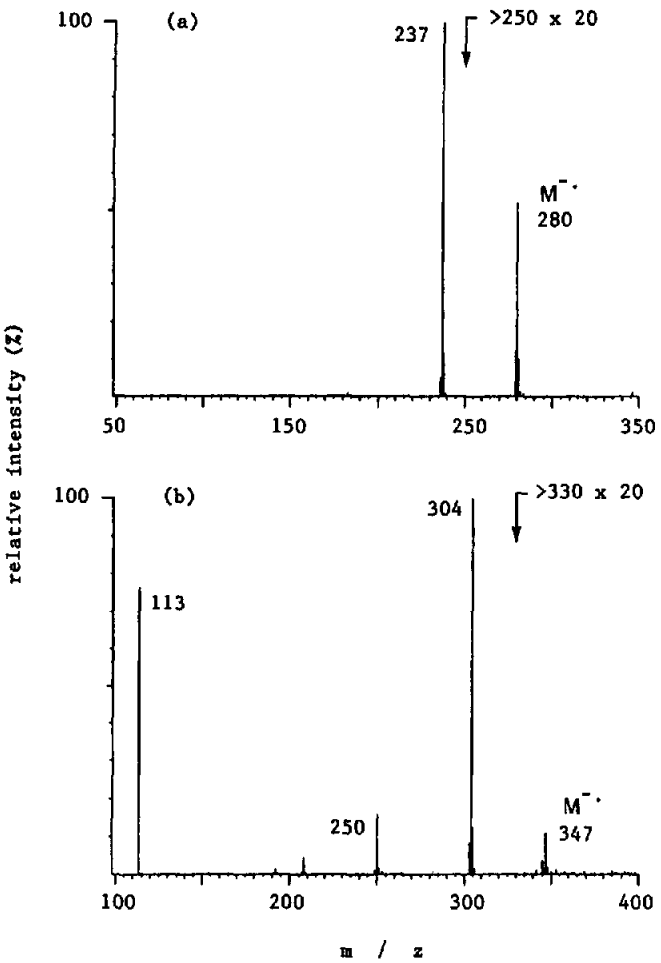

Fig. 3. ECNICI mass spectra of the TFA derivatives of (a) [$^2H_5$]phenacetin and (b) [$^2H_4$]paracetamol.

immediately prior to analysis by GC–MS, and, using this procedure, no further difficulties were encountered.

Deuterated analogues of phenacetin and paracetamol were then synthesised for use as internal standards in the assay. Phenacetin labelled with five deuterium atoms in the ethoxy group ([$^2H_5$]phenacetin) was made by reacting paracetamol with pentadeuteroethyl iodide. The ECNICI mass spectrum of the TFA derivative of [$^2H_5$]phenacetin (Fig. 3a) was, as expected, analogous to that of the unlabelled compound, with the small molecular ion at *m/z* 280 and base peak at *m/z* 237 being five mass units higher than for the non-deuterated compound. Paracetamol containing three deuterium atoms in the acetyl group ([$^2H_3$]paracetamol) was synthesised by trideuteroacetylation of 4-aminophenol. The ECNICI

mass spectrum of the TFA derivative of [$^2H_3$]paracetamol, however, indicated that deuterium exchange was occurring during the derivatisation procedure. This was presumably due to enolisation of the trideuteroacetyl group catalysed by trifluoroacetic acid present in the derivatising reagent. An alternative analogue of paracetamol labelled with four deuterium atoms in the aromatic ring ([$^2H_4$]paracetamol) was commercially available and the ECNICI mass spectrum of the TFA derivative of this compound (Fig. 3b) showed that, with this analogue, the problem of deuterium exchange, did not exist.

Standards for the measurement of phenacetin and unconjugated paracetamol, containing phenacetin (0–50 ng), [$^2H_5$]phenacetin (20 ng), paracetamol (0–1.5 μg) and [$^2H_4$]paracetamol (1 μg) in small volumes of methanol, were prepared. These solutions were evaporated to dryness, derivatised and then analysed by GC–MS. Because there was much more paracetamol and [$^2H_4$]paracetamol than phenacetin and [$^2H_5$]phenacetin in the standards, the mass spectrometer was tuned to monitor the weak molecular ions of the TFA derivatives of paracetamol and [$^2H_4$]paracetamol at *m/z* 343 and 347, respectyively. This resulted in a similar SIM response being obtained for the TFA derivatives of the four compounds and allowed phenacetin and paracetamol to be measured in a single chromatogram. Over the ranges shown above, the unextracted standard curves for phenacetin and paracetamol were linear with intercepts close to zero (Table I).

TABLE I

STANDARD CURVE PARAMETERS FOR THE MEASUREMENT OF PHENACETIN, UNCONJUGATED PARACETAMOL AND TOTAL PARACETAMOL

Compounds	Standard curve[a]		
	Slope (*m*)	y-Intercept (*c*)	Correlation coefficient (*r*)
Unextracted (methanol solutions)			
Phenacetin (ng)	0.047	0.003	0.999
Unconjugated paracetamol (μg)	1.004	0.016	1.000
Total paracetamol (ng)			
m/z 246 and 250	0.00548	0.010	0.999
m/z 300 and 304	0.00368	0.001	1.000
Extracted (from drug-free plasma)			
Phenacetin (ng)	0.048	−0.010	1.000
Unconjugated paracetamol (μg)	0.999	0.020	0.999
Total paracetamol (ng)			
m/z 246 and 250	0.00557	−0.006	1.000
m/z 300 and 304	0.00368	0.004	1.000

[a] $y = mx + c$, where y is the measured peak-area ratio of compound to internal standard and x is the amount of compound present in the sample.

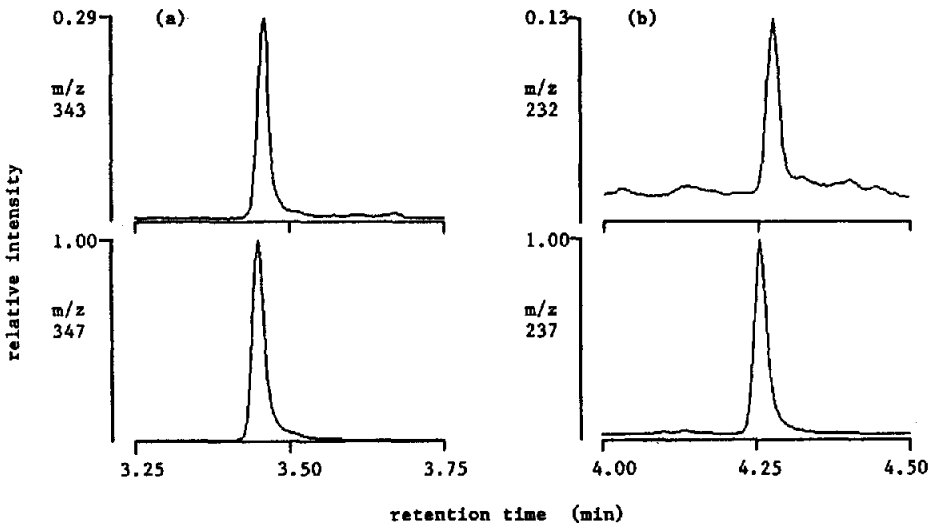

Fig. 4. SIM traces for the analysis of (a) unconjugated paracetamol (0.5 μg/ml) and (b) phenacetin (5 ng/ml) in plasma.

A simple diethyl ether extraction was used to isolate phenacetin and unconjugated paracetamol from plasma and, after derivatisation, analysis by GC–MS gave SIM traces that were free of any interference. Recovery through the extraction, assessed by comparison of internal standard peak areas in extracted samples with those in unextracted standards, was ~80% for phenacetin and ~50% for paracetamol. Standards identical to those used for preparation of the unextracted standard curves were extracted from drug-free plasma (typical SIM traces are shown in Fig. 4). The slopes and intercepts of the extracted standard curves were the same as those of the unextracted standard curves (Table I) and so the latter were used for the routine analysis of samples. The accuracy and precision for measurement of phenacetin (4 ng/ml of plasma) were 3.9 ng/ml ± 2.6% (mean ± S.D., $n = 6$) and for paracetamol (0.5 μg/ml of plasma) 0.48 μg/ml ± 1.4% (mean ± S.D., $n = 6$). The limit of detection for measurement of phenacetin was 1 ng/ml of plasma.

After ingestion of phenacetin, some unconjugated paracetamol appears in the bloodstream but the metabolite is present mainly as its sulphate and glucuronide conjugates. Cysteine and mercapturic acid conjugates have also been identified in plasma but these are only present at significant levels in subjects suffering severe liver damage following paracetamol overdosage [16]. Hence, to measure total paracetamol levels, plasma was incubated with a mixed sulphatase–glucuronidase enzyme preparation to hydrolyse the paracetamol conjugates in plasma, prior to extraction.

Standards for total paracetamol analysis containing paracetamol (0–300 ng)

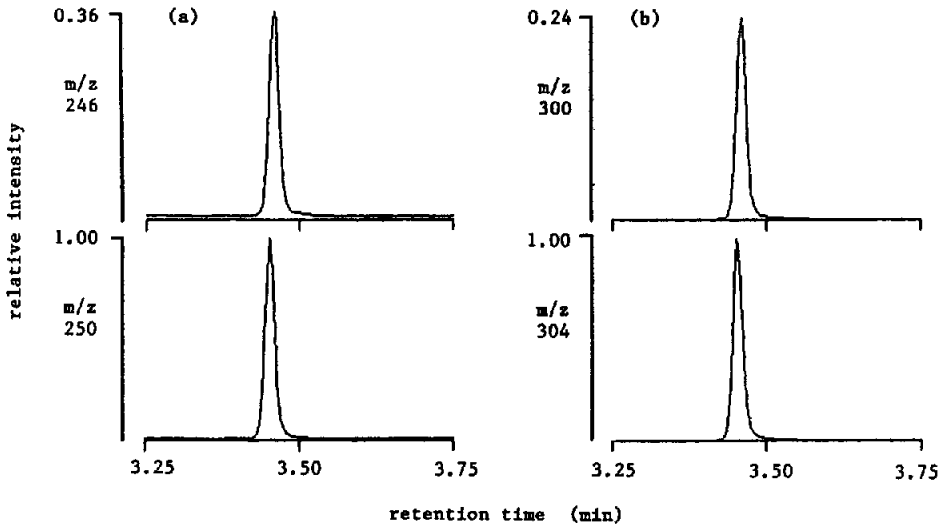

Fig. 5. SIM traces for the analysis of total paracetamol (0.6 μg/ml) in plasma recording ion pairs (a) *m/z* 246 and 250 and (b) *m/z* 300 and 304.

and [$^2H_4$]paracetamol (200 ng) were prepared, derivatised and analysed by GC–MS as described above. The mass spectrometer was tuned to monitor two pairs of negative ions, at *m/z* 246 and 250 and at *m/z* 300 and 304, and the unextracted standard curve obtained with each pair of ions was linear with an intercept close to zero (Table I). Standards identical to those used for preparation of the unextracted standard curves were extracted from drug-free plasma following enzyme hydrolysis (typical SIM traces are shown in Fig. 5) and the SIM traces obtained were found to be free of any interference. The slopes and intercepts of

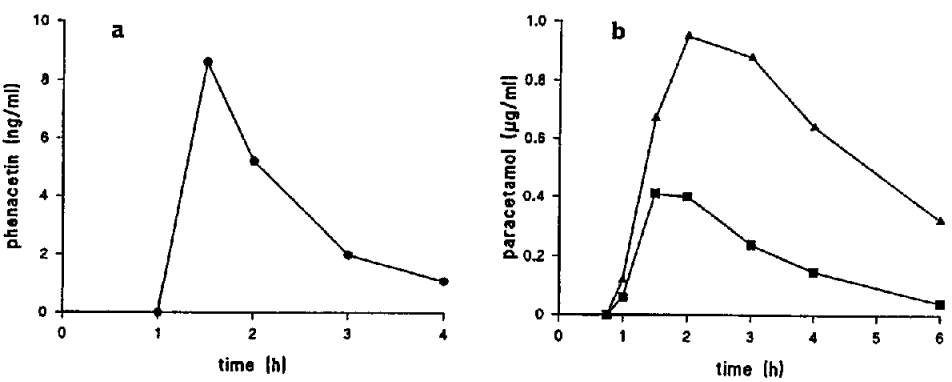

Fig. 6. Plasma levels of (a) phenacetin (●) and (b) unconjugated (■) and total (▲) paracetamol in a subject receiving a 100-mg oral dose of phenacetin.

the extracted standard curves were the same as those of the unextracted standard curves (Table I) and so the latter were used for the routine analysis of samples. The accuracy and precision for measurement of paracetamol (0.5 μg/ml of plasma) were 0.49 μg/ml ± 1.7% (mean ± S.D., $n = 6$) when monitoring ions m/z 246 and 250, and 0.52 μg/ml ± 2.4% (mean ± S.D., $n = 6$) when monitoring ions m/z 300 and 304.

When the assay described here was applied to the analysis of large numbers of plasma samples, a chromatographic problem was encountered. After approximately twenty injections had been made on the capillary column, the peak shapes of the TFA derivatives of phenacetin and, to a greater extent, paracetamol began to degrade and exhibit pronounced tailing. This problem was resolved by raising the GC oven temperature to 275°C for a minimum period of 30 min, after which the chromatographic behaviour of the compounds returned to normal.

In a preliminary experiment, levels of phenacetin, unconjugated and total paracetamol in the plasma of a subject given a single 100-mg oral dose of phenacetin were determined using the assay methods described above. While the peak plasma concentration of phenacetin was found to be less than 10 ng/ml (Fig. 6a), total paracetamol 2 h after drug administration was present at a concentration of almost 1 μg/ml (Fig. 6b). Unconjugated paracetamol made up approximately 50% of total paracetamol at time points up to 1.5 h but decreased rapidly as a percentage of the total after this (Fig. 6b). These results are in close agreement with those reported by other workers [7].

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