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ORIGINAL RESEARCH

Analysis of the glutathione conjugate of paracetamol in human liver microsomal fraction by liquid chromatography mass spectrometry

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ABSTRACT: An assay based on microbore liquid chromatography–positive ion electrospray mass spectrometry with selected ion recording has been developed for the measurement of the glutathione conjugate of paracetamol (acetaminophen) in human hepatic microsomal incubations. A deuterated analogue of the glutathione conjugate was synthesized for use as an internal standard and extraction was carried out on Oasis™ solid phase extraction columns. The limit of quantification of the glutathione conjugate was 10 ng/incubation and the intra-assay coefficient of variation at this concentration was 6.8%. Copyright © 2001 John Wiley & Sons, Ltd.

INTRODUCTION

Paracetamol (also known as acetaminophen) is a widely available, non-prescription drug used for its analgesic and antipyretic properties. While safe when used at normal doses, the compound is hepatotoxic when large amounts are ingested and centrilobular necrosis occurs in both animals and man (Boyd and Bereczky, 1966; Prescott *et al.*, 1971). This toxicity is a result of the formation of the reactive metabolite *N*-acetyl-*p*-benzoquinoneimine (NABQI) (Mitchell *et al.*, 1973). At normal dosage this metabolite is removed by rapid reaction with glutathione [Jollow *et al.*, 1974; Figure 1(a)] but at higher concentrations glutathione reserves are depleted and binding to cellular macromolecules occurs (Jollow *et al.*, 1973).

Our interest in paracetamol toxicity stemmed from the possibility of using paracetamol as a prodrug in gene-directed enzyme prodrug therapy. The aim of this technique, which has potential in the treatment of cancer, is to selectively target tumour cells by transfecting them with a gene encoding an enzyme capable of activating a prodrug. The production of a toxic metabolite then kills the transformed cells while leaving other healthy cells undamaged. The formation of NABQI from paracetamol is mediated by the cytochrome P450 mixed function oxidase enzyme system in the liver and it has been shown, using human liver microsomal fraction and specific inhibitory antibodies, that several different isoforms of cytochrome P450 can contribute to the production of this metabolite (Raucy *et al.*, 1989; Patten *et al.*, 1993). These groups estimated NABQI production *in vitro* by measuring the formation of the glutathione conjugate of paracetamol (paracetamol GSH). Previous work has shown that this is the single product of the reaction between NABQI and glutathione in the rat both *in vivo* and *in vitro* and that there is no evidence of deactivation of NABQI by oxidation of glutathione (Smith and Mitchell, 1985; Porubek *et al.*, 1987).

For our work, it was necessary to identify the major form or forms of P450 responsible for the production of NABQI at toxic concentrations of the drug and an assay for paracetamol GSH was therefore required. High performance liquid chromatography (HPLC) with UV and electrochemical detection has been used previously to measure this compound (Raucy *et al.*, 1989; Patten *et al.*, 1993) but this methodology, while sensitive, can lack specificity. Liquid chromatography–mass spectrometry is a sensitive and highly specific analytical technique that is ideally suited to the analysis of polar and involatile drug conjugates and accurate quantification can be obtained with the use of a stable isotope labelled analogue of the analyte as an internal standard. A method based on microbore liquid chromatography–positive ion electrospray mass spectrometry using a deuterated analogue of paracetamol GSH as internal standard has therefore been developed and is described here.

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Abbreviations used: ESI, electrosprayionization; NABQI, *N*-acetyl-p-benzoquinoneimine; SIR, selected ion recording; SRM, selected reaction monitoring.

EXPERIMENTAL

Chemicals. Microsomal fractions were isolated from samples of human liver as described elsewhere (Boobis *et al.*, 1990) and a pooled sample prepared from 10 individual fractions. The tissue, from the human liver bank maintained in the Section on Clinical Pharmacology at Imperial College School of Medicine, was obtained from liver transplant cut-downs, liver resections and kidney transplant donors. Tissue was stored frozen at −70°C until isolation of the microsomal fraction and all samples were histologically normal. The use of samples for these studies had local Research Ethical Committee permission and, where appropriate, coroner's approval. Silver nitrate, paracetamol, glutathione (reduced form) and NADPH were obtained from Sigma-Aldrich Ltd (Poole, Dorset, UK). Paracetamol GSH was a kind gift from Sanofi Synthelabo, while *N*-(4-hydroxyphenyl-2,3,5,6-^{2}H)acetamide ([$^2H_4$]-paracetamol) was purchased from MSD Isotopes (Montreal, Quebec, Canada). Oasis™ extraction cartridges were obtained from Waters Ltd (Watford, UK). Acetonitrile and formic acid were of HPLC grade while other organic solvents were of analytical grade. Water was generated by a Milli-Q water purification system (Millipore, Watford, UK) and had a resistivity of 18.2 MΩ cm.

Synthesis of [$^2H_3$]-paracetamol GSH

Silver oxide. Sodium hydroxide solution (2 M, 100 mL) was added with stirring to silver nitrate solution (400 mM, 25 mL) in a conical glass flask at room temperature. The mixture was agitated for a further 10 min, then the precipitated silver oxide was isolated by filtration through a glass sinter. The product was washed with water (125 mL), acetone (125 mL), then diethyl ether (125 mL) and dried overnight under vacuum (yield 1.16 g, 5 mmol, 100%).

[$^2H_4$]-NABQI. Freshly prepared silver oxide (700 mg, 3 mmol) was added to a solution of [$^2H_4$]-paracetamol (15.5 mg, 100 μmol) in chloroform (50 mL) in a conical glass flask. The flask was purged with nitrogen, stoppered and the flask contents stirred for 2 h at room temperature. The reaction mixture was then passed through a glass sinter and the filtrate used immediately in the next reaction.

[$^2H_3$]-paracetamol GSH. Glutathione (reduced form) in 0.07 M potassium phosphate buffer pH 7.4 (4 mM, 33 mL) was placed in a conical glass flask and the vessel purged with nitrogen. [$^2H_4$]-NABQI (∼100 μmol) in chloroform (50 mL) was then added dropwise with vigorous stirring over a period of 10 min. The flask was stoppered and the reaction mixture stirred for a further 1 h at room temperature. The reaction mixture was then transferred to a separating funnel, the chloroform layer discarded and the aqueous phase washed twice with chloroform. The aqueous phase was taken to dryness by lyophilization, the residue redissolved in a small volume of water and [$^2H_3$]-paracetamol GSH purified by preparative HPLC. Chromatography was carried out on a μBondapak phenyl column (25 cm × 4.6 mm i.d.) with acetonitrile/water/formic acid (5/95/0.1, v/v/v) as solvent. At a flow rate of 1.5 mL/min, the retention time of [$^2H_3$]-paracetamol GSH was 5.5 min (yield 28.4 mg, 61.9 μmol, 62%).

Incubation conditions. Incubation mixtures consisting of human hepatic microsomal fraction (2 mg/mL, 50 μL), NADPH (10 mg/mL, 10 μL), glutathione (reduced form, 150 mM, 20 μL) and 100 mM sodium phosphate buffer pH 7.4 (80 μL) were warmed to 37°C in a shaking waterbath. The reaction was started by the addition of paracetamol dissolved in 100 mM sodium phosphate buffer pH 7.4 (20 or 100 mM, 40 μL) to a final concentration of 4 or 20 mM and the samples were then incubated for 25 min at 37°C. The reaction was stopped by the addition of 43% phosphoric acid (20 μL), [$^2H_3$]-paracetamol GSH in water (10 μg/mL, 30 μL) was added to each incubation mixture and the samples were placed on ice.

Sample extraction. Samples were diluted with water (750 μL) and, following centrifugation (10 min, 10,000***g***), were applied to Oasis™ SPE columns (60 mg sorbent mass, 3 mL reservoir volume) which had been preconditioned with methanol (1 mL) and water (1 mL). The columns were washed with water (1 mL), after which paracetamol GSH and [$^2H_3$]-paracetamol GSH were eluted with methanol (1 mL). Methanol eluates were evaporated to dryness under nitrogen, the residues reconstituted in acetonitrile/water/formic acid (2/97/1, v/v/v, 15 μL) and aliquots (10 μL) injected onto the microbore HPLC column.

Standard curve. Eight standards in glass vials were prepared from stock solutions of paracetamol GSH (5 μg/mL methanol) and [$^2H_3$]-paracetamol GSH (10 μg/mL methanol). The standards all contained [$^2H_3$]-paracetamol GSH (300 ng) as well as paracetamol GSH (0, 10, 25, 50, 100, 250, 500 and 750 ng). After evaporation to dryness under nitrogen, standards were reconstituted in acetonitrile/water/formic acid (2/98/0.1, v/v/v, 15 μL) and aliquots (10 μL) injected onto the microbore HPLC column.

Microbore HPLC–electrospray ionisation mass spectrometry. An HPLC system consisting of a Waters 616 liquid chromatography pump and 600S flow controller (Waters, Watford, UK), and a Rheodyne model 8125 injector (Rheodyne, Cotati, CA, USA) fitted with a 20 μL sample loop, was used. Standards or incubation extracts were chromatographed on a YMC phenyl column of dimensions 15 cm × 1 mm i.d. (Hichrom, Theale, UK) fitted with a YMC phenyl guard cartridge (1 cm × 1 mm i.d.), under isocratic conditions using a mobile phase of acetonitrile/water/formic acid (10/90/0.1, v/v/v). At a flow rate of 70 μL/min, the retention times of paracetamol GSH and [$^2H_3$]-paracetamol GSH were 4.6 min. The column was connected directly to the electrospray interface of a VG Quattro II triple quadrupole mass spectrometer (Micromass, Altrincham, UK). This instrument was operated in the positive ion electrospray mode with a source temperature of 70°C, a capillary voltage of 3.2 kV and a cone voltage of 30 V. For selected ion recording (SIR) work, the mass spectrometer was tuned, with Q1 acting as mass filter at unit resolution, to monitor ions *m/z* 457 and *m/z* 460, the protonated molecular ions of paracetamol GSH and [$^2H_3$]-paracetamol GSH, respectively. The dwell time per ion was 0.25 s with a 0.02 s interchannel delay and peak area ratios were determined. For detection of paracetamol GSH and [$^2H_3$]-paracetamol GSH by selected reaction monitoring (SRM), the mass spectrometer was set to transmit the parent–product ion pairs *m/z* 457 → 328, *m/z* 457 → 382, *m/z* 460 → 331 and *m/z* 460 → 385 with a dwell time of 0.12 s for each and a 0.02 s interchannel delay. Argon was used as collision gas at a pressure of 9×10^{-4} mbar and the collision

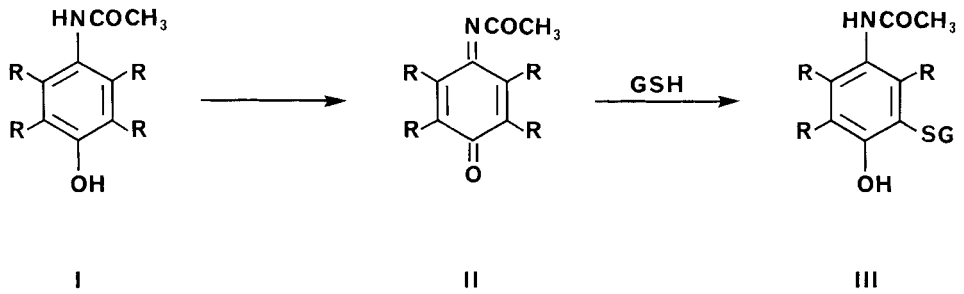

Figure 1. (a) R = H. The oxidation of paracetamol (I) to *N*-acetyl-*p*-benzoquinoneimine (NABQI, II) and the reaction of NABQI with glutathione (GSH) to give the glutathione conjugate of paracetamol (paracetamol GSH, III). (b) R = ^{2}H. The synthesis of [$^2H_3$]-paracetamol GSH (III) from [$^2H_4$]-paracetamol (I).

energy was set at 35 eV. Data acquisition and processing were performed with Masslynx software.

RESULTS

The synthesis of the internal standard, [$^2H_3$]-paracetamol GSH, is summarized in Fig. 1(b). Oxidation of [$^2H_4$]-paracetamol was carried out with freshly prepared silver oxide and the [$^2H_4$]-NABQI formed was used immediately to make the deuterated adduct. The identity of the product was confirmed by positive ion electrospray ionization (ESI) mass spectrometry with the mass spectrum of the compound containing only a protonated molecular ion of *m/z* 460. The yield of [$^2H_3$]-paracetamol GSH from [$^2H_4$]-paracetamol was 62%.

Paracetamol GSH and [$^2H_3$]-paracetamol GSH were chromatographed on a phenyl reversed-phase microbore column under isocratic conditions. At a flow rate of 70 μL/min, the adduct and its deuterated analogue had a retention time of 4.6 min. Paracetamol GSH generated a simple positive ion ESI mass spectrum with a protonated molecular ion of *m/z* 457 and so SIR of ions *m/z* 457 and 460 formed the basis of a quantitative assay for the glutathione adduct. Using standards containing paracetamol GSH (0–750 ng) and [$^2H_3$]-paracetamol GSH (300 ng), an unextracted standard curve was obtained from plotting intensity ratios I_{457}/I_{460} against amount of paracetamol GSH. This was found to be linear ($y = 0.00323x + 0.005$, $r^2 = 0.999$) and the presence of $<0.5\%$ paracetamol GSH in the deuterated internal standard resulted in the intercept of the standard curve passing essentially through the origin.

A solid phase extraction (SPE) procedure based on the use of Oasis™ cartridges was developed. No methanol was added to the water used in the wash step as this was found to result in partial elution of the analyte. Recovery of known amounts of paracetamol GSH from blank incubation mixtures that had no NADPH present and had not been incubated at 37°C was assessed by comparing the response of equivalent unextracted standards and was estimated to be $>90\%$. The peak area ratios of extracted standards were not significantly different from those of corresponding unextracted standards, indicating that there was no paracetamol GSH present in blank incubation mixtures and that there was no chromatographic interference. The slopes of extracted standard curves differed by $<3\%$ from unextracted standard curves and so the latter were used for the routine analysis of samples. The limit of detection of paracetamol GSH in microsomal incubation mixtures was 3 ng (extracted standards containing this amount of material gave intensity ratios that were approximately three times the blank value). The limit of quantification for the measurement of paracetamol GSH was set at 10 ng, the lowest point on the standard curve, where for extracted standards the accuracy and precision were 10.43 ± 0.72 ng (mean ± s.d., $n = 6$, CV 6.8%).

The enzymic activity of the pooled microsomal fraction was measured at two substrate concentrations, 4 and 20 mM paracetamol. At both concentrations, production of paracetamol GSH was linear up to 40 min and so an incubation period shorter than this (25 min) was used for kinetic measurements. At 4 mM paracetamol, 343 ± 15 pmol paracetamol GSH/mg microsomal protein/min incubation time was produced while at 20 mM paracetamol the enzymic activity was 584 ± 33 pmol product/mg/min (mean ± s.d., $n = 6$).

DISCUSSION

The assay for paracetamol GSH described here was based on the use of liquid chromatography mass spectrometry with a deuterated analogue of the analyte as an internal standard. Huggett and Blair have described the synthesis of NABQI and paracetamol GSH from paracetamol (Huggett and Blair, 1983a, b) and the same synthetic route was used here to make [$^2H_3$]-paracetamol GSH [Fig. 1(b)]. The analogue was synthesized in high yield from deuterated paracetamol and its use as an internal standard gave the assay a limit of quantification of 10 ng.

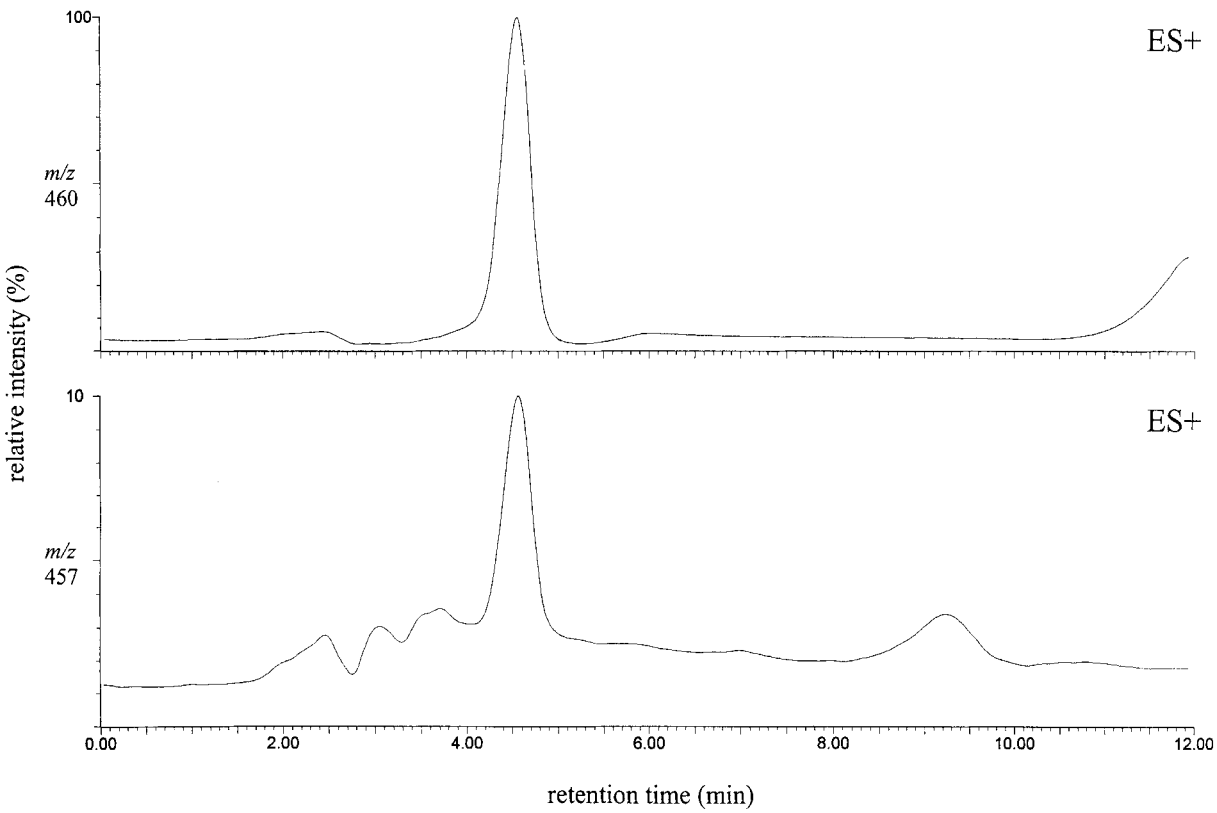

Figure 2. Positive ion electrospray (ES+) selected ion recording chromatograms for the analysis of the glutathione conjugate of paracetamol (25 ng) in a microsomal incubation (*m/z* 457, paracetamol GSH, retention time 4.6 min; *m/z* 460, [$^2H_3$]-paracetamol GSH, retention time 4.6 min).

During method development, it was discovered that good chromatographic behaviour for paracetamol GSH was only obtained when the pH of the mobile phase was $\leq$3. This suggests that suppression of ionisation of the two carboxyl groups within the glutathione part of the molecule (pK_a 3–4) is necessary to maintain good peak shape. Consequently a mobile phase containing 0.1% formic acid (pH 2.8) was used and incubation extracts were reconstituted in solvent containing 1% formic acid.

Both SIR and SRM mass spectrometry were assessed for the detection and measurement of paracetamol GSH. With argon as collision gas, collision-induced dissociation of the protonated molecular ion of paracetamol GSH gave two product ions (*m/z* 328 and *m/z* 382) of almost equal intensity, corresponding to cleavage of the amide bonds within the glutathione part of the molecule. The protonated molecular ion of [$^2H_3$]-paracetamol GSH fragmented to give the analogous ions *m/z* 331 and *m/z* 385. Consequently SRM of the parent–product ion pairs *m/z* 457 $\rightarrow$ 328 and *m/z* 460 $\rightarrow$ 331, or *m/z* 457 $\rightarrow$ 382 and *m/z* 460 $\rightarrow$ 385, could be used as the basis of a quantitative assay for paracetamol GSH. However, extracts of microsomal incubations gave SIR chromatograms that were free of chromatographic interference (a typical chromatogram is shown in Fig. 2). Hence SIR was used for the routine analysis of samples and SRM mass spectrometry, where greater specificity is obtained by the recording of product ions produced by collision-induced dissociation, was not required.

Rates of formation of paracetamol GSH were measured at substrate concentrations that have been associated with hepatotoxicity. It has been reported that plasma concentrations of paracetamol greater than 1 mM, 4 h after consumption of an overdose of the drug, result in a high incidence of severe hepatic damage (Prescott, 1980, 1984) and so enzyme activity was initially measured at concentrations of 4 and 20 mM paracetamol. The figures obtained for rates of formation of paracetamol GSH by the pooled microsomal fraction at 4 mM (343 pmol paracetamol GSH / mg microsomal protein / min incubation time) and 20 mM (584 pmol / mg/min) were similar to those reported previously by other workers. Raucy *et al.* (1989), when measuring formation of the cysteine conjugate of paracetamol, found activities of 130 and 378 pmol/mg/min at concentrations of 1 and 10 mM paracetamol, respectively, while Patten *et al.* (1993) have reported an activity of 570 pmol/mg/min when using a substrate concentration of 0.5 mM paracetamol.

While previous workers have used inhibitory anti-

bodies to estimate the contribution of various isoforms of P450 to the formation of NABQI (Raucy *et al.*, 1989; Patten *et al.*, 1993), similar investigations can be undertaken with specific chemical inhibitors of enzyme activity. A number of specific chemical inhibitors have been identified over the last decade that are now widely used to define the contribution of different isoforms of cytochrome P450 in metabolic processes *in vitro* (Chang *et al.*, 1994; Bourrie *et al.*, 1996; Sai *et al.*, 2000). Further studies using these compounds, together with investigations into the variability of P450 activity in microsomal fractions from different tissue samples, are currently in progress.

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