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Determination of paracetamol and its four major metabolites in mouse plasma by reversed-phase ion-pair high-performance liquid chromatography

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ABSTRACT

A reversed-phase ion-pair high-performance liquid chromatographic method has been used for the separation of paracetamol and its four major metabolites (glucuronide, sulphate, cysteine and mercapturate conjugates) in mouse plasma samples. An ODS column was used and the mobile phase consisted of an aqueous solution of 0.01 *M* tetrabutylammonium chloride and 0.01 *M* Tris buffered to pH 5.0 with phosphoric acid, with methanol as the organic solvent. The gradient elution started with 30% methanol. After a delay of 0.5 min the methanol concentration was increased linearly to 75% over 7.5 min. The column was returned to the initial conditions after a delay of 1 min. A methanol solution of theophylline was added to the mouse plasma sample, centrifuged and immediately injected into the chromatographic system. The advantages of this method include good and rapid separation (last metabolite detected at 6.86 min), well resolved peaks, only a small amount of sample required for assay, adequate precision (no coefficient of variation was greater than 10% for paracetamol metabolites) and a high sensitivity (particularly for unchanged paracetamol and the cysteine conjugate).

INTRODUCTION

Paracetamol, acetaminophen or N-acetyl-*p*-aminophenol, is a widely used analgesic agent that causes liver necrosis in humans and experimental animals when high doses are ingested or administered [1,2]. Studies on the metabolism of acetaminophen have shown two major pathways of elimination. One of these involves glucuronidation and sulphation (glucuronide and sulphate conjugates) and the other, oxidation, forms cysteine and mercapturic acid conjugates. Evidence strongly implicates a role for minor oxidised metabolites in the hepatotoxic reaction caused by paracetamol. The production of irreversible liver damage in overdose situations has made the study of paracetamol metabolism of toxicological importance [3–7].

Different procedures, such as spectrophotometry and gas chromatography, have been used to determine the concentration of unchanged paracetamol and its metabolites in biological samples. The best results have been obtained using high-performance liquid chromatography (HPLC). This method has proved to be rapid, sensitive and precise in the quantitative determination of drugs, in particular that of paracetamol and its derivatives [3,8–12].

The purpose of this study was to develop a reliable method by which plasma concentrations of paracetamol and its metabolites may be determined. These compounds range from relatively non-polar unconjugated paracetamol to the cor-

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responding highly water-soluble sulphate and glucuronide conjugates. Reversed-phase HPLC has traditionally been used to separate paracetamol metabolites. At present, ion-pair chromatography is the method which achieves the best results [3,13–15].

Few methods for the determination of paracetamol metabolites in plasma samples from experimental animals have been reported [4,6,8,9,16–19]. These are usually limited to the determination of glucuronide and sulphate metabolites and all four metabolites are rarely quantified.

As a result of this study, a simple, highly sensitive ion-pair reversed-phase HPLC method is proposed for the separation of paracetamol and its sulphate, glucuronide, cysteine and mercapturate conjugates, each of which was present at various concentrations in mouse plasma. Additional advantages of the method include its speed, accuracy and the small amount of sample required for assay (about a minimum of 50 μl of plasma).

EXPERIMENTAL

Apparatus

A Beckman (San Ramon, CA, USA) liquid chromatograph equipped with a solvent delivery system with two Model 110B pumps, an analogue interface module 406, a Model 166 ultraviolet detector and a Model 210A injector with 20-μl sample loop was used in conjunction with a computer (GOLD system). Separation was performed at ambient temperature on a 25 cm × 4 mm I.D. Ultrasphere ODS (5 μm particle size) column. The mobile phase consisted of an aqueous solution of 0.01 *M* tetrabutylammonium chloride (TBA) and 0.01 *M* tris(hydroxymethyl)-aminomethane (Tris), with the pH adjusted to 5.0 with phosphoric acid, and methanol.

Materials

Paracetamol, the internal standard, theophylline and TBA were obtained from Sigma (St. Louis, MO, USA). Paracetamol glucuronide, sulphate, cysteine and mercapturate conjugates were generously donated by Winthrop Labs. Production Division (Fawdon, Newcastle upon Tyne, UK). Methanol, Tris and distilled, deionized water were purchased from Merck (Darmstadt, Germany). The methanol and water were vacuum-degassed before use.

Procedures

Sample preparation. Plasma samples were obtained from two groups of ten Swiss mice under 4-h fasting conditions during which single doses of 500 mg/kg (15 mg/ml) paracetamol were administered either intraperitoneally or orally. The animals were killed by decapitation 1 h (ten animals) and 2.5 h (ten animals) after the administration of paracetamol and were then exanguinated. Plasma samples were stored frozen at −80°C before analysis.

A 100 μg/ml methanol solution of theophylline was added to the plasma at a ratio of 1:1. This mixture was allowed to precipitate for at least 10 min and centrifuged twice at 11 000 *g* 6–7 min to pellet the precipitated proteins. The mixtures were filtered through 0.45-μm HV Millipore cellulose filters and 20-μl samples were injected immediately into the chromatographic system.

Chromatography. The gradient elution started with 30% methanol passing through the column at a flow-rate of 1.5 ml/min. After a delay of 0.5 min the methanol concentration was increased lineary to 75% over 7.5 min. The column was returned to 30% methanol after a delay of 1 min. Before the next sample was injected, 15 min elapsed. The variable-wavelength detector was set at 254 nm.

Quantitation. Calibration graphs were prepared by adding known amounts of paracetamol and its four metabolites to human serum diluted 1:1 with water (Table I). All reference samples

TABLE I

CONCENTRATIONS OF PARACETAMOL AND ITS METABOLITES USED FOR CALIBRATION GRAPHS

Analyte	Initial concentration (mg/ml)	Concentration found (μg/ml)		
		High	Medium	Low
Paracetamol	5.00	500.0	250.0	50.0
Glucuronide	11.34	567.0	283.5	56.7
Sulphate	15.64	156.4	78.2	15.6
Cysteine	4.89	122.4	61.2	12.2
Mercapturate	7.14	142.8	71.4	14.3

were analysed in duplicate. Peak-height ratios of paracetamol and its metabolites to the internal standard, theophylline, were used to construct standard graphs. All standard graphs were calculated by linear regression analysis of peak-height ratios *versus* the concentration of paracetamol metabolites.

RESULTS AND DISCUSSION

The retention times obtained were 1.96, 2.48, 2.98, 3.32, 6.64 and 6.86 min for paracetamol cysteine, unchanged paracetamol, internal standard, paracetamol glucuronide, paracetamol mercapturate and paracetamol sulphate, respectively.

Figs. 1–3 shows representative chromatograms of standards of paracetamol and its metabolites, a test plasma sample and a blank plasma sample. The results indicate that there was good separation and well resolved peaks.

The calibration graphs were all linear for a wide range of concentrations, which allows the analysis of undiluted samples. Coefficients of determination, r^2, of 0.97 or better for all metabolites indicate a precise and accurate calibration (Table II). Both the areas and peak-height ratios were used to calculate the standard graphs. When standard graphs were calculated using peak-height ratios, greater coefficients of determination for all metabolites were obtained. This finding has been also reported by other workers [4,9,13,19].

The highest sensitivity, estimated as the slope of the calibration graphs, was for the unchanged paracetamol and cysteine conjugate and the lowest for the glucuronide and mercapturate conjugates (Table II).

The limit of detection was estimated with reference to a signal-to-noise ratio of about 2, using 20-μl injections of serial dilutions of water-diluted human serum to which standards had been added. The results were as follows: paracetamol, 0.6 μg/ml (12 ng); glucuronide, 4.5 μg/ml (90 ng); sulphate, 0.6 μg/ml (12 ng); cysteine, 1.0 μg/ml (20 ng); and mercapturate, 2.1 μg/ml (42 ng). These limits of detection are similar to those obtained by Bhargava *et al.* [19] in plasma samples, but higher than those obtained by Wilson *et al.*

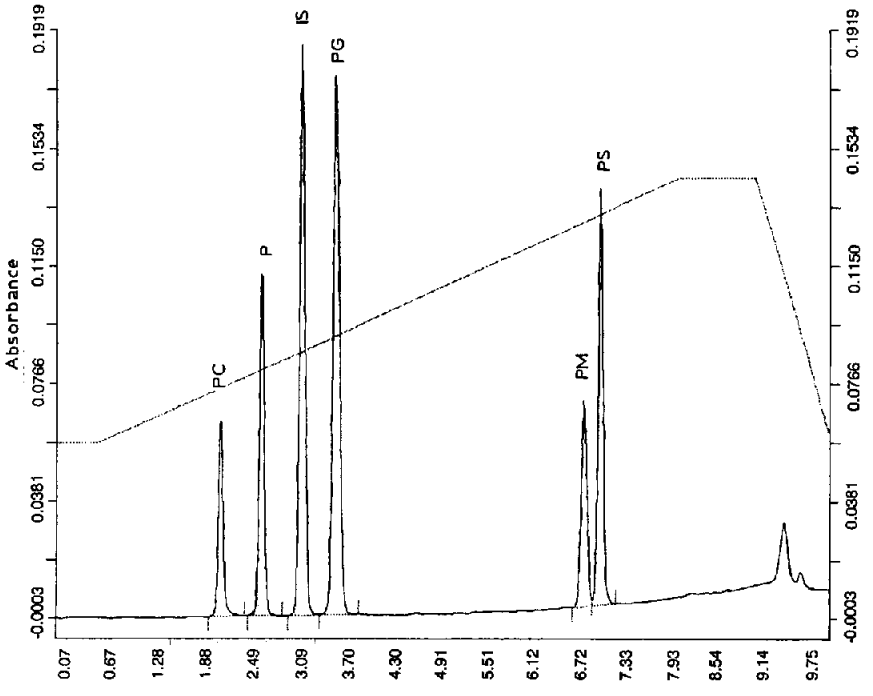

Fig. 1. Chromatogram of aqueous standards of paracetamol (P) and its metabolites [paracetamol glucuronide (PG), paracetamol sulphate (PS), paracetamol cysteine (PC) and paracetamol mercapturate (PM)] at low concentrations. IS = Internal standard (theophylline).

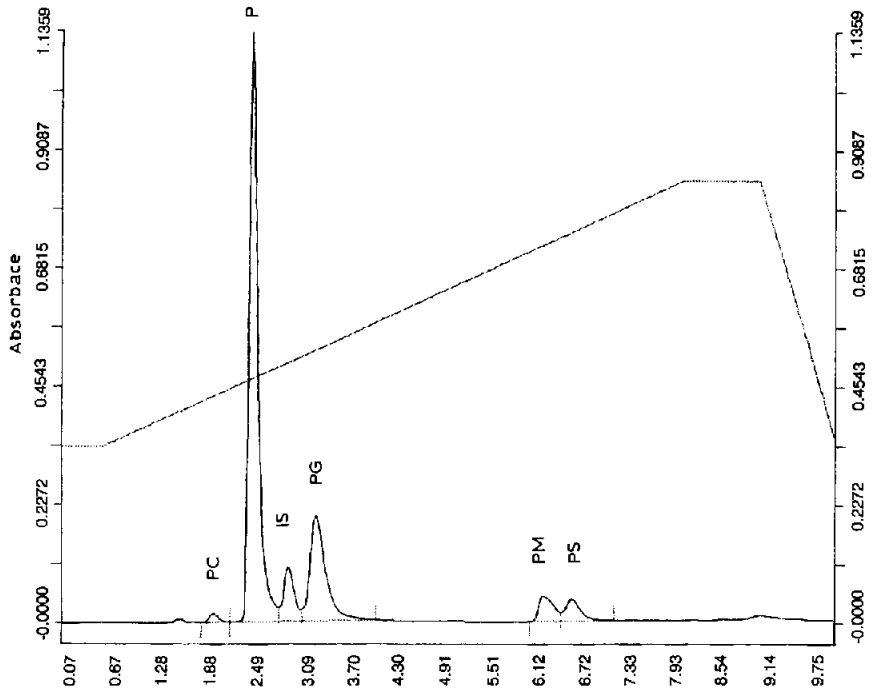

Fig. 2. Chromatogram of a mouse plasma sample with 448.1 μg/ml paracetamol (P), 233.3 μg/ml paracetamol glucuronide (PG), 34.5 μg/ml paracetamol sulphate (PS), 9.5 μg/ml paracetamol cysteine (PC) and 53.9 μg/ml paracetamol mercapturate (PM).

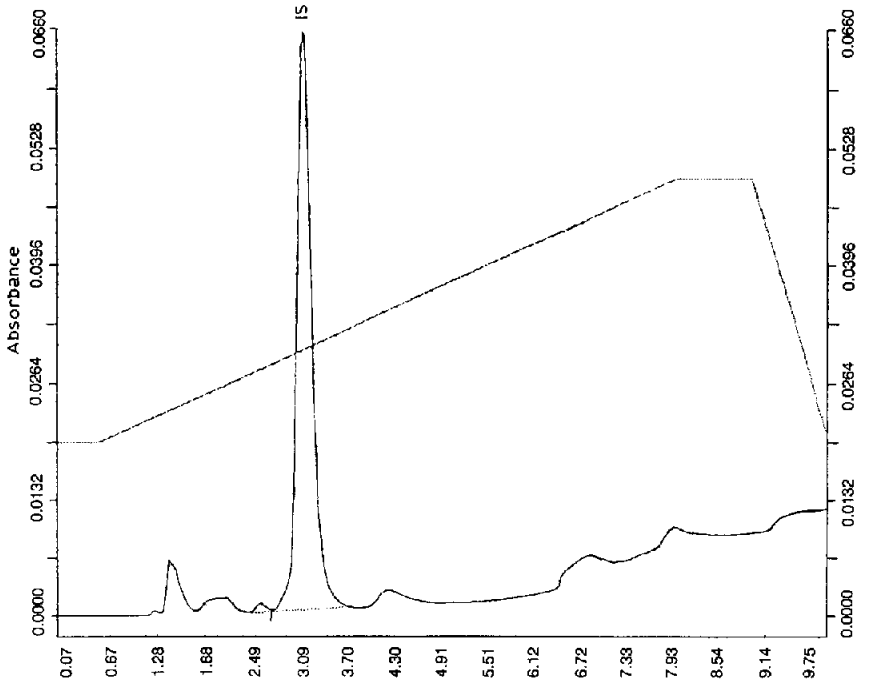

Fig. 3. Chromatogram of a blank plasma sample. IS = Internal standard.

TABLE II

PARAMETERS OF THE CALIBRATION GRAPHS AND SENSITIVITY OF THE METHOD

Analyte	Slope (sensitivity)	y-Intercept[a]	r^2
Paracetamol	0.0231	0.381	0.9985
Glucuronide	0.0082	0.010	0.9766
Sulphate	0.0108	0.039	0.9952
Cysteine	0.0147	0.031	0.9993
Mercapturate	0.0081	0.029	0.9959

[a] y = peak-height ratio; x = concentration.

[3] in urine samples using UV and amperometric detection. Although the use of amperometric detection results in a more efficacious procedure, it is more time-consuming as different dilutions of urine are required for the detection of each analyte.

Accuracy was estimated by the percentage difference between theoretical concentrations of the standards in the calibration graphs of each compound and the corresponding concentrations calculated by regression analysis (Table III). The small deviation of the values obtained provides additional support for the validity of the method.

Inter-assay and intra-assay precision were calculated by the method of Rodbard [20]. A pool of mouse plasma at two different drug concentrations (high and low) from previously quantified plasma samples was used. Both high and low drug concentrations were analysed in triplicate over three days (Table IV). The coefficients of variation were within acceptable limits. The highest values were for paracetamol sulphate and glucuronide conjugates and may be attributed to a more marked dependence of the acidic forms on pH and other factors.

TABLE III

DIFFERENCE (%, IN PARENTHESES) BETWEEN THE AVERAGE OF THE ESTIMATED CONCENTRATION IN THE STANDARD GRAPHS AND THE CORRESPONDING THEORETICAL CONCENTRATION

See Table I for levels of calibration.

Compound	Concentration found (μg/ml)		
	High	Medium	Low
Paracetamol	495.5 (0.9)	259.4 (3.8)	45.1 (9.7)
Glucuronide	567.3 (0.1)	274.0 (14.7)	66.0 (23.3)
Sulphate	154.3 (3.0)	82.1 (5.0)	13.8 (11.8)
Cysteine	121.7 (0.6)	62.7 (2.4)	11.5 (6.4)
Mercapturate	141.1 (2.8)	74.7 (4.6)	12.7 (10.8)

TABLE IV

INTER- AND INTRA-ASSAY COEFFICIENTS OF VARIATION (C.V) OF PARACETAMOL AND ITS METABOLITES

Compound	Concentration (μg/ml)	Intra-assay C.V. (%)	Inter-assay C.V. (%)
Paracetamol	114.1	5.22	2.69
	393.8	1.81	6.54
Glucuronide	102.0[a]	2.66	9.40
Sulphate	20.5[a]	8.33	3.13
Cysteine	7.4[a]	4.78	5.82
Mercapturate	41.7[a]	3.03	4.62

[a] Mean value of high and low concentration.

The efficacy of the method may be improved by extracting paracetamol and its metabolites from different biological fluids, prior to injection into the chromatographic system, with organic solvents [12], ion-pair liquid–liquid extraction [21] or solid-phase extraction [22]. These procedures eliminate interferences and increase the sensitivity of the method. In none of the above-mentioned references was a study of the extraction of the four metabolites of paracetamol made.

Determinations of urinary metabolites other than glucuronide, sulphate, cysteine and mercapturate conjugates by ion-pairing HPLC [5,13], a combination of UV and electrochemical detection [3] and a fully automated liquid chromatographic method [11] have been reported. However, in none of these studies were references made to the possibility of determining these or other metabolites in plasma samples. In this study, other metabolites of paracetamol could not be quantified because the corresponding standards were not available.

Although the four metabolites of paracetamol in plasma samples have not previously been quantified, Jung and Zafar [9] developed a micro-

HPLC assay procedure for the determination of paracetamol and glucuronide and sulphate conjugates, and Bhargava *et al.* [19] described the simultaneous determination of the mercapturate conjugate. Ion-pairing systems were not used and the samples were treated by precipitation with perchloric acid before injection into the chromatographic system. In addition, references to extraction were not made. Both studies reported similar coefficients of variation, although the calibration graphs included a lower concentration range than that described in this study.

CONCLUSIONS

According to the coefficients of variation and r^2 obtained in this procedure, the acid compounds are most susceptible to variations in pH and other factors. Thus, the quantitative determination of these paracetamol conjugates was not as realiable as that of others which were precisely determined. This method is capable of quantitatively determining all four metabolites in mouse plasma, possibly as a result of the high doses of paracetamol used. It is a useful tool for monitoring drugs and studying the metabolism and pharmacokinetics of paracetamol. The determination of cysteine and mercapturate conjugates in plasma may contribute to a better understanding of the hepatotoxic effects of paracetamol.

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