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Assay of paracetamol and its metabolites in urine, plasma and saliva of children with chronic liver disease

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Abstract

A high-performance liquid chromatographic (HPLC) assay of paracetamol and its metabolites is described. The method for quantifying the metabolism of paracetamol in children with chronic liver disease and the good correlation between plasma and salivary concentrations of paracetamol is demonstrated. Despite an increasing bias between the two methods with increasing concentration of paracetamol, it is concluded that salivary assay is satisfactory for characterising paracetamol pharmacokinetics in the group of patients studied.

Keywords: Assay; Liver disease; Paracetamol metabolism; Pharmacokinetics

1. Introduction

Aspirin is no longer recommended as an analgesic or antipyretic for children because of reported associations with Reye's syndrome [1–3]. As a result, paracetamol is now the standard analgesic/antipyretic for such patients. However, it is well known that paracetamol, which is extensively metabolised by the liver mainly by conjugation to the sulphate and glucuronide, may be converted to a highly toxic oxidative metabolite, *N*-acetyl-*P*-benzoquinoneimine (NABQI) when administered in overdose [4–6]. The metabolite, which is formed through the intermediary of cytochrome P450 mixed function oxidase, is usually detoxified by combining with glutathione [7]. However, when paracetamol is administered in overdose, glutathione stores are depleted and toxicity ensues. Therefore, it is important to know how children with chronic liver disease metabolise the drug. Young children (less than 12 years old) and neonates excrete more of an oral dose of paracetamol as the sulphate than do adults [8]. Indeed, in the young (<12 years), conjugation to the sulphate is the predominant elimination pathway, while glucuronidation is more important in older subjects [8].

As part of a study to investigate the safety of paracetamol in children with liver disease, a high-performance liquid chromatographic (HPLC) method was developed for assaying

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paracetamol and its metabolites in body fluids. Moreover, the possible use of salivary samples for defining the drug's pharmacokinetics in such children was evaluated. If there is a direct correlation between plasma and salivary concentrations in such children with liver disease, then this would enable us to avoid venipuncture, which is always an unpleasant experience in this population of patients, in subsequent studies. Good correlations between plasma and salivary paracetamol concentrations have previously been reported [9], but no comparable work has been reported on the metabolites. Good correlations between plasma and salivary concentrations have been reported for a number of drugs including tolbutamide [10] and phenytoin [11].

2. Experimental

2.1. Study protocol

Thirteen children with chronic hepato-biliary disease and aged between 0.6 and 12.0 years with normal renal function were investigated in this study which was approved by the Ethics Committee of The Queen's University of Belfast and conducted at the Royal Belfast Hospital for Sick Children, Belfast. Informed consent was obtained from the parents and if appropriate from the children themselves following a full explanation of what was involved. Following an overnight fast, paracetamol suspension (Calpol®) was given as a single oral dose of 10 mg kg^{-1} followed by 10 ml kg^{-1} of orange juice. Food was withheld for 1 h after dosing and blood samples collected at intervals over periods of up to 9.0 h. For six patients (patients 6, 8, 9, 10, 11 and 13) saliva samples and for a further six (patients 1, 2, 6, 7, 8 and 10) the total volume of urine voided over a 36 h period were also collected. Five patients were restudied after a period of 2.5–10 months. Of those, four (patients 1, 3, 6, and 8) provided blood samples and the fifth (patient 7) saliva samples only. The study protocol stresses that the patients were volunteers. Reflecting the practical difficulties of ethical studies in childhood, it was impossible to always match the urine, plasma and saliva samples.

2.2. HPLC

Paracetamol, paracetamol glucuronide, paracetamol sulphate, paracetamol cysteine and para-cetamol mercapturate were the generous gift of Sterling Winthrop, Newcastle, UK. 3-Acetaminophen and 4-fluorophenol were from Sigma, Poole, UK. Orthophosphoric acid 85% for HPLC, perchloric acid 60% GPR and potassium hydroxide pellets, Analar, were purchased from BDH Chemicals Ltd., Poole, UK. Acetonitrile far UV and HPLC grade methanol were from Lab-Scan Ltd., Dublin, Ireland, and Pellicular ODS, 37–53 μm was from Whatman International Ltd., UK. HPLC grade water was prepared in our laboratory using the Milli Q® system.

The HPLC system consisted of an HPLC pump (Beckman model 110 A), a WISP autoinjector (Waters Inc. model 712), a data integrator (Hewlett-Packard model 3390 A), a precolumn (5 cm × 4.6 mm ID packed with Pellicular ODS, 37–53 μm), a variable wavelength UV detector (Kratos Analytical Instruments model Spectroflow 773) and a column heater (Jones Chromatography). Two columns were used: a μ-Bondapak® C-18 reversed-phase column (10 μm, 30 cm × 3.9 mm ID) and a Spherisorb® ODS2 reversed phase (5 μm, 25 cm × 4.6 mm ID) column. All assays were conducted with the column thermostated at 25 °C.

2.3. Standard curves

Standard curves were prepared using standard solutions containing 0.625, 1.25, 2.5, 5, 10, 20, and 40 μg ml^{-1} of paracetamol and each of paracetamol glucuronide, paracetamol sulphate, para-cetamol cysteine and paracetamol mercapturate, as well as 10 μg ml^{-1} of 3-acetaminophen, the internal standard. The standard solutions were prepared from drug-free urine and stock solutions containing 1 mg ml^{-1} of one of the paracetamol compounds. The stock solution was serially diluted with urine to produce the required range of concentrations. Comparison of the results using different urine concentrations produced identical results. For the plasma standard curves the stock solutions were serially diluted with drug-free plasma. The saliva standard curves were prepared similarly, but using saliva as diluent during the serial dilution and omitting the paracetamol metabolites

as none were detectable in significant amounts.

Calibration curves were constructed by plotting the peak–height ratio of the drug to the internal standard versus the concentration of the drug.

3. Results and discussion

3.1. Assay developement

Urine samples

Initial attempts at analysing paracetamol and its metabolites were based on the method reported by Miners et al. [12], using a reversed-phase μ-Bondapak C-18 column and a mobile phase consisting of acetonitrile–orthophosphoric acid (20 mmol) (2.5:97.5, v/v) (pH* 4.7) adjusted with potassium hydroxide. Normal urine components were found to interfere with paracetamol glucuronide and sulphate.

Adjustment of pH enabled resolution of the sulphate peak, but not the glucuronide peak, from the urine components (Fig. 1), while altering the acetonitrile composition caused peak broadening for one or more of the peaks, in particular the mercapturate conjugate.

The method reported by Sommers et al. [13] based on a Spherisorb ODS2 reversed phase column was than tried, but using a mobile phase consisting of acetonitrile and 20 mmol orthophosphoric acid adjusted to various pH values. Acetonitrile–orthophosphoric acid (20 mmol) (4:96, v/v) (pH 3.5) produced the most satisfactory separation for urine samples (Fig. 2), and 3-acetaminophen was found to be a suitable internal standard.

Plasma samples

Using the method developed for urine samples, clear separations were obtained for paracetamol and its metabolites (glucuronide, sulphate, cysteine and mercapturate) from normal plasma components. These were all eluted within the first 3 min after sample injection. However, the mobile phase composition was adjusted to methanol–acetonitrile–orthophosphoric acid (20 mmol) (2:4:94, v/v/v) adjusted to pH 3.2 to resolve the glucuronide peak, increase peak sharpness and reduce sample run-time (Fig. 3). 3-Acetaminophen was again a suitable internal standard.

Saliva samples

The assay developed for urine samples was satisfactory for assay of paracetamol and its metabolites in saliva. However, as none of the paracetamol metabolites were detectable in significant amounts, a change in acetonitrile composition of the mobile phase to 6% and omitting methanol based on the assay developed by Sommers et al. [13] improved peak sharpness and shortened assay time (Fig. 4).

In the assays of plasma and saliva, the internal standard was prepared in 30% perchloric acid which acted as a protein precipitant when one part was added to nine parts of standard or test solutions. All solutions were centrifuged at 3000 rev min^{-1} for 15 min after addition of the internal standard, and the supernatants were analysed.

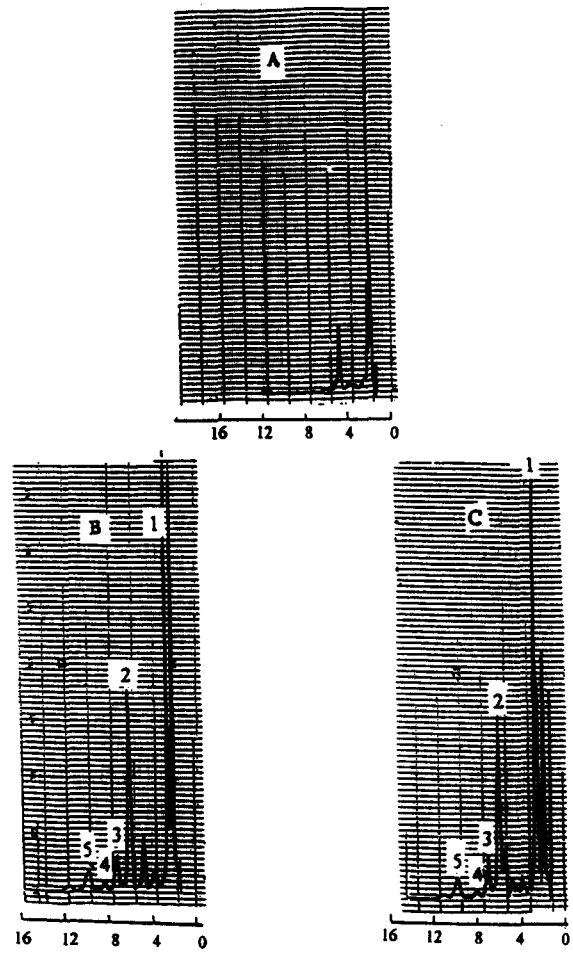

Fig. 1. Typical chromatograms obtained from: (A) drug-free urine; (B) and (C) urine sample collected overnight from a healthy adult volunteer after administration of 1 g of paracetamol and analysed by the method reported by Miners et al. [12] at pH 4.7 and 5.3, respectively, of the mobile phase. Peaks are (1) paracetamol glucuronide, (2) paracetamol sulphate, (3) paracetamol cysteine, (4) paracetamol and (5) paracetamol mercapturate.

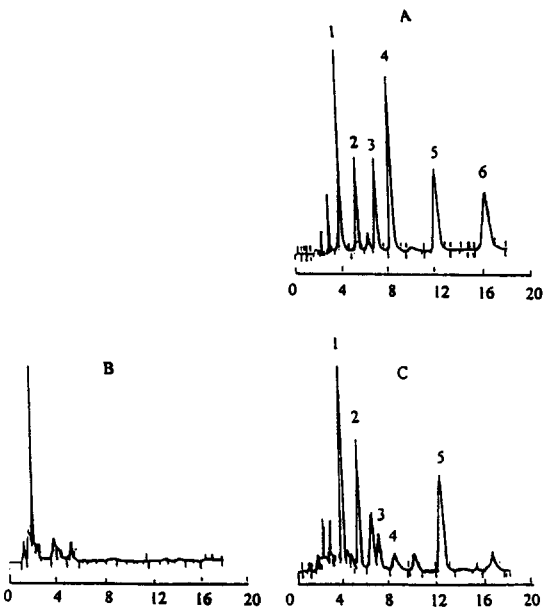

Fig. 2. Typical chromatograms obtained from: (A) drug-free urine spiked with authentic compounds and internal standard; (B) drug-free urine; (C) urine sample collected from one of the patients studied. Peaks are (1) paracetamol glucuronide, (2) paracetamol sulphate, (3) paracetamol cysteine, (4) paracetamol, (5) internal standard, 3-actaminophen and (6) paracetamol mercapturate.

Standard curves in urine over the range 0.625–40 μg ml^{-1} were linear for all five compounds ($r^2 > 0.996$), and the coefficients of variation based on five samples for 20 μg ml^{-1} and 2.5 μg ml^{-1} solutions were 1.34% and 2.04%, respectively, for paracetamol. The corresponding figures were 1.42% and 1.81% for paracetamol glucuronide, 1.72% and 2.20% for paracetamol sulphate, 1.48% and 2.09% for paracetamol cysteine, and 1.73% and 2.20% for paracetamol mercapturate. For these metabolites, the retention times were 3.8, 5.3, 7.0 and 16.2 min, respectively. Paracetamol and 3-acetaminophen had retention times of 8.3 and 12.1 min, respectively. The inter- and intra-day coefficients of variation were less than 2.56% in all cases, with the worst reproducibility being observed, as expected, at the lowest concentration (0.625 μg ml^{-1}).

For plasma, the retention time for 3-acetaminophen was 9.3 min, and those for paracetamol, paracetamol glucuronide, paracetamol sulphate, paracetamol cysteine and paracetamol mercapturate were 6.6, 3.5, 4.5, 5.6 and 12.2 min respectively. The coefficients of variation for standard 20 μg ml^{-1} and 2.5 μg ml^{-1} solutions based on five samples were 1.98 (2.26)%, 2.23 (2.83)%, 1.80 (1.95)%, 1.47 (1.64)% and 2.04 (2.68)%, respectively, for paracetamol and its metabolites as listed above.

In saliva, no metabolite was observed at any quantifiable concentration. The paracetamol and 3-acetaminophen retention times were 6.0 and 8.3 min, respectively and based on five samples; the coefficients of variation using 20 μg ml^{-1} and 2.5 μg ml^{-1} solutions of paracetamol were 1.83% and 2.61%, respectively.

In all assays, the lowest determinable concentration of each of the compounds assayed was less than 0.20 μg ml^{-1}.

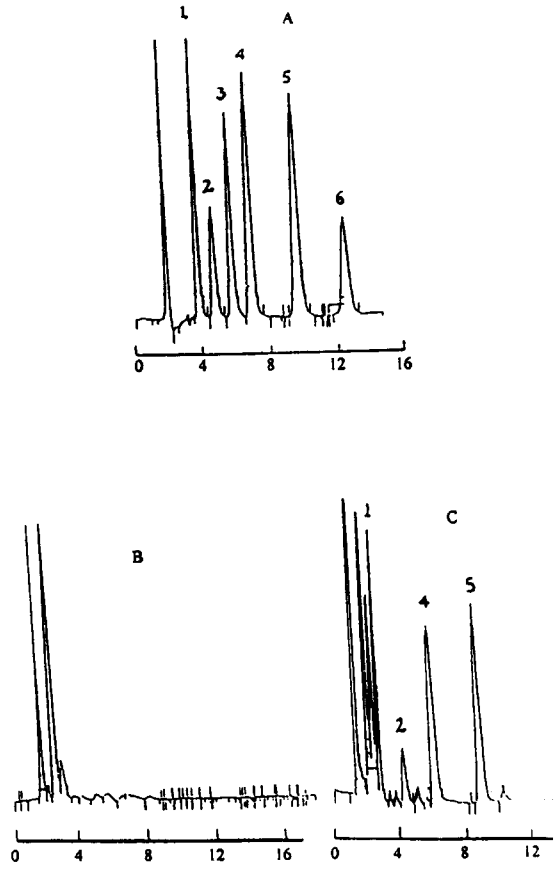

Fig. 3. Typical chromatograms obtained from: (A) drug-free plasma spiked with authentic compounds and internal standard; (B) drug-free plasma; (C) plasma sample collected from one of the patients studied. Peaks are (1) paracetamol glucuronide, (2) paracetamol sulphate, (3) paracetamol cysteine, (4) paracetamol, (5) internal standard, 3-actaminophen and (6) paracetamol mercapturate.

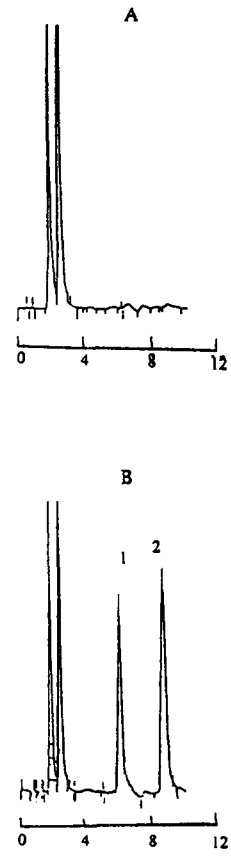

Fig. 4. Typical chromatograms obtained from: (A) drug-free saliva; (B) drug-free saliva spiked with paracetamol and internal standard. Peaks are (1) paracetamol and (2) internal standard, 3-acetaminophen.

Fig. 5 shows the plasma concentration–time and salivary concentration–time profiles of paracetamol in the six patients from whom

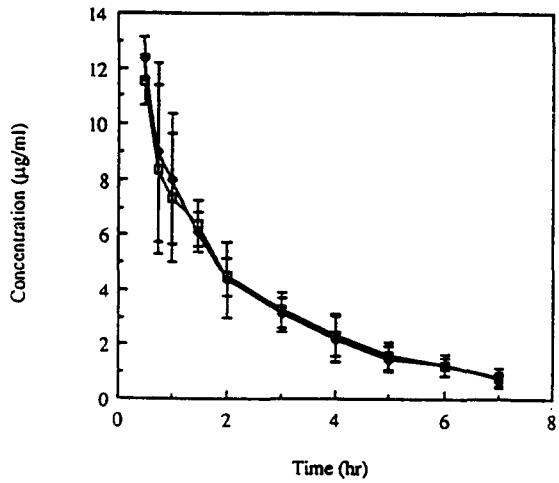

Fig. 5. Average plasma (—⊡—) and salivary (—◆—) paracetamol concentration–time profile of all six patients. Each point represents mean ± SD.

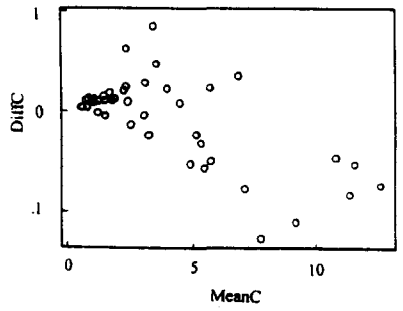

Fig. 6. A plot of the mean plasma/saliva conentration (Mean C) against the difference in observed concentrations (DiffC). Units of measurement μg ml^{-1}.

paired data were obtained. There was clearly concordance between the results. To check for bias, the mean plasma/saliva concentration was plotted against the difference in observed concentrations as shown in Fig. 6. It can be seen that although a plot (Fig. 7) of the plasma versus salivary concentrations appeared linear ($r^2 = 0.991$), there was an obvious relationship between the difference in concentrations and the size of the measurements. In fact, a regression of the difference (y) on the mean (x) showed a significant linear relationship ($r^2 = 0.456$ and $p < 0.001$), which could be described by the equation $y = 0.268 - 0.0883x$. Therefore, while salivary concentrations can be used to define paracetamol pharmacokinetics, there was a bias which increased with the magnitude of the concentration being measured.

To illustrate the usefulness of the method described, the urinary concentrations of paracetamol and of its metabolites were assayed in six children, and Table 1 gives the percentage recovery of the individual components in urine samples pooled over 36 h.

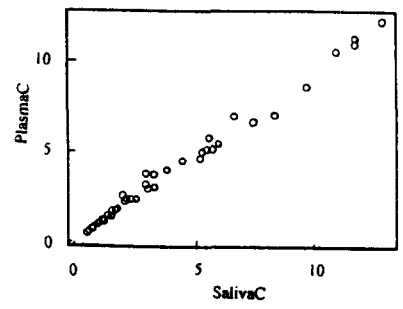

Fig. 7. Correlation between (PlasmaC) and saliva concentrations (SalivaC) of paracetamol (μg ml^{-1}) in all six patients.

Table 1
Urinary recovery of paracetamol and its glucuronide, sulphate, cysteine and mercapturic acid conjugates in six subjects pooled over 36 h

Patients	APAP (%)	APAP-G (%)	APAP-S (%)	APAP-C (%)	APAP-M (%)	% of total excreted	Ratio of glucuronide to sulphate
1	2.78	41.89	42.33	5.25	7.23	99.47	0.99
2	3.44	50.43	32.28	7.20	6.29	99.64	1.56
3	3.37	45.70	17.70	5.23	7.65	79.65	2.58
4	1.78	38.24	44.95	6.29	7.13	98.39	0.85
5	3.31	37.83	26.97	6.00	7.24	81.35	1.40
6	1.30	37.92	37.63	4.45	6.15	87.45	1.01
Mean ± SE	2.66 ± 0.37	42 ± 2.10	33.64 ± 4.20	5.74 ± 0.39	6.95 ± 0.24	90.99 ± 3.81	1.40 ± 0.26

APAP, paracetamol; APAP-G, paracetamol glucuronide; APAP-S, paracetamol sulphate; APAP-C, paracetamol cysteine; APAP-M, paracetamol mercapturate.

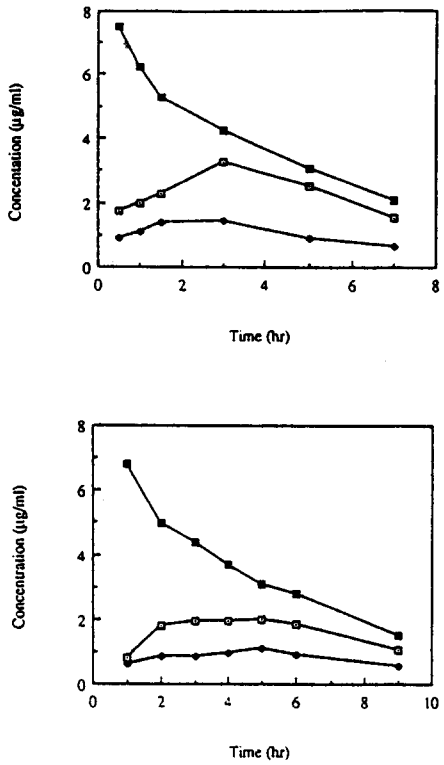

Fig. 8. Plasma concentration of paracetamol (— □ —) and its glucuronide (— ⊡ —) and sulphate (— ◆ —) conjugates after administration of a single oral dose of 10 mg kg^{-1} in one patient with severe biliary atresia on two occasions about 6 months apart.

For plasma samples, the method described also provided a useful means of characterising the pharamacokinetic profile of paracetamol and its glucu-ronide and sulphate conjugates as shown in Fig. 8.

4. Conclusion

The methods described enable the quantification of paracetamol and its metabolites in plasma, urine and saliva. However, no measureable levels of paracetamol conjugates were detectable in the saliva of our paediatric patients. In plasma, only the glucuronide and the sulphate appeared in measurable amounts. While there was a good correlation between plasma and saliva levels of paracetamol, the saliva assay gave a positive bias relative to the plasma assay which increased with the concentration being measured. However, the differences would be unlikely to make any significant difference to the magnitude of the pharmacokinetic parameters being estimated.

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