Pergamon

0731-7085(94)00085-9

Journal of Pharmaceutical & Biomedical Analysis, Vol. 12, No. 12, pp. 1563–1572, 1994

0731-7085/94 $7.00 + 0.00

The estimation of paracetamol and its major metabolites in both plasma and urine by a single high-performance liquid chromatography assay

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Abstract: Many analytical methods exist for the assay of paracetamol in biological fluids, including colorimetry with chemical derivatization, direct spectrophotometry, chromatographic methods and immunoassays. Their development has been largely driven by the needs of clinical toxicology requiring the rapid, reliable and highly specific estimation of paracetamol in plasma samples to determine the need for antidote therapy. However, for *in vivo* metabolism studies, a specific assay method which can provide measurements of paracetamol and its metabolites in both plasma and urine is desired. A reversed-phase HPLC method with UV detection at 254 nm was developed to fulfil these requirements. The assay involves minimum sample preparation with a relatively short run time. The solvent system involves a simple isocratic elution with a composition of 0.1 M potassium dihydrogen orthophosphate–acetic acid–propan-2-ol, (100:0.1:0.75, v/v/v). The reproducibility of the assay was high with an inter-assay RSD of 0.2–1.7% for urinary paracetamol concentrations of 5–500 $\mu g\ ml^{-1}$ and 0.1–3.3% for plasma concentrations between 5 and 25 $\mu g\ ml^{-1}$. A similarly high degree of precision was found for the glucuronide, sulphate, cysteine and mercapturate metabolites of paracetamol. The same assay can be used to analyse both plasma and urine samples and thus was employed for studies on the metabolism of paracetamol in healthy subjects and in patients with various diseases.

Keywords: *Paracetamol; HPLC.*

Introduction

Paracetamol is a widely used and normally very safe analgesic. Its metabolism has been well elucidated as parallel pathways of oxidation and conjugation [1]. It is an ideal drug for the study of factors influencing the different routes of drug metabolism in man [2] and has also been utilized for the study of gastric emptying [3]. Paracetamol is metabolized primarily by conjugation with glucuronide and sulphate when given in therapeutic doses. A small proportion is oxidized by microsomal cytochrome P450 enzymes yielding a highly reactive intermediate which is normally conjugated with glutathione, and eventually excreted as the cysteine and mercapturate conjugates. The measurement of these two oxidative metabolites serves as an indicator of the extent of metabolic activation and capacity for glutathione synthesis. Inter-subject and ethnic differences in the metabolic activation of paracetamol have been studied for toxicological and pharmacogenetic purposes [2]. For studies on the metabolism of paracetamol, the assay method used must be able to measure the individual metabolites of paracetamol, as well as the unchanged drug.

Many analytical methods exist for the assay of paracetamol in biological fluids, including colorimetry [4, 5], spectrophotometry, gas–liquid [6–11], thin layer [12] and high-performance liquid chromatography [13–18], and immunoassays [19, 20]. However, the development of these assays have been largely driven by the needs of clinical toxicology, and they may not suit the requirements of detailed studies on paracetamol metabolism and related pharmacokinetics. Our present studies involve the estimation of paracetamol *and* its main metabolites in multiple samples of *both* plasma and urine. Thus sample preparation needed to be minimized and specific method developed. An HPLC method was favoured because of the specificity and sensitivity offered, and the possibility of semi-automation. This paper reports the successful development of a specific HPLC assay for the simultaneous estimation of

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paracetamol and its four major metabolites, namely glucuronide, sulphate, cysteine and mercapturate, in both plasma and urine. Results of validation studies including those on the accuracy, sensitivity and recovery of this assay are presented as well as a specimen set of data from six normal healthy volunteers.

Materials and Methods

Paracetamol, paracetamol glucuronide, paracetamol sulphate, paracetamol cysteine and paracetamol mercapturate for reference standards were kindly donated by Sterling Winthrop Research and Development Division (Alnwick, UK). Potassium dihydrogen orthophosphate, propan-2-ol and 60% perchloric acid were obtained from E. Merck, and acetic acid from BDH Chemical Ltd. The internal standard 2-acetamidophenol (AAP) was obtained from Sigma. Water was purified for HPLC by the Milli Q Water Purification System (Millipore, Bedford, MA, USA).

Instrumentation

The HPLC system consisted of a Waters 501 pump which delivered the mobile phase, degassed by sonification, via a filter to the system. The mobile phase then passed to a Waters Intelligent Sample Processor 712 (WISP) which is an automatic sample injection module.

Prior to passing through the analytical column, the solvent was passed through a Waters Guard-PAK precolumn containing the same packing material as the column. The column used was an 8 mm × 10 cm Waters Radial-PAK Cartridge. Packing material was Nova-Pak C_{18}, particle size 5 μm. The cartridge was housed in a Waters Radial Compression Module (RCM 8 × 10).

From the column, the eluate then passed through a Waters 440 UV detector connected to a Waters 745B Data Module to produce the chromatograms and peak areas.

Collection and storage of samples

Blood samples were collected into 10-ml heparinized tubes, centrifuged for 10 min at 1400*g* and the plasma separated for storage.

Urine was collected into containers containing chloroform (30 ml for 24-h urine collection) to help prevent breakdown of the conjugates, especially the glucuronide. Urine volume was recorded and a 20 ml aliquot was stored. Prior to analysis, all samples were stored at −20°C. Paracetamol and its conjugates are stable in plasma and urine at this temperature for a period of years [21].

Chromatographic conditions

For the estimation of paracetamol, paracetamol glucuronide, paracetamol sulphate, paracetamol cysteine and paracetamol mercapturate in urine and plasma, the following chromatographic conditions are used: the mobile phase consists of 0.1 M potassium dihydrogen orthophosphate, acetic acid and propan-2-ol in the ratio 100:0.1:75 by volume, at a flow rate of 1.5 ml min^{-1}. The pressure of the system is maintained at 1000 psi. The detection wavelength is 254 nm.

Urine assay

Preparation of standards and test samples for the urine assay. For estimating paracetamol and its metabolites in urine, standard solutions of paracetamol (25, 250, 1250 and 2500 μg ml^{-1}) in blank urine with the internal standard acetamidophenol (AAP, 250 μg ml^{-1}) were prepared.

The paracetamol standards and samples were prepared in the following way for analysis. To 500 μl of standard or sample, 1000 μl of water and 1000 μl of AAP solution were added resulting in a 5-fold dilution. The injection volume was 3 μl. Calibration standards were run with each batch of samples for calculation.

Validation of the urine assay. A stock solution of paracetamol in blank urine was serially diluted to give concentrations ranging from 5 to 500 μg ml^{-1}. Samples were assayed as described and over a period of a few days were analysed four times. The same was done for all the paracetamol metabolites studied using standard solutions.

Calculation of results for the urine assay. The concentrations of paracetamol and its metabolites in the samples were calculated in paracetamol equivalents from the peak area ratio of paracetamol or its conjugate to the internal standard AAP. The peak area alone instead of the peak area ratio was also used (see discussion under validations of plasma assay). Standard curves were calculated by the least-squares regression analysis of the peak area ratio (or peak area) vs drug concen-

tration. The reciprocal of the slope of the regression line is the factor by which the peak area ratios (or peak areas) are multiplied to give the paracetamol concentrations in $\mu g\ ml^{-1}$.

Only enough pure paracetamol was available in sufficient quantities for running as standards with each batch of samples. This could not be done for the metabolites due to the minute quantities of the latter available. Thus the values obtained are expressed as paracetamol equivalents. The UV absorbances of the glucuronide and sulphate conjugates of paracetamol are not the same as that of paracetamol and correction factors of 0.909 for the glucuronide conjugate and 0.945 for the sulphate conjugates in urine at 254 nm were used for calculating the concentrations of these conjugates [21]. No correction factor was used for the cysteine and the mercapturate conjugates [21].

Plasma assay

Preparation of standards and test samples for the plasma assay. For estimating therapeutic concentrations of paracetamol and its metabolites, spiked plasma standards containing 5, 15 and 25 $\mu g\ ml^{-1}$ of paracetamol were prepared. These were run after every fourth sample. An internal standard was not used in the plasma assay (see under *Results of plasma assay*). The calibration standards and samples were prepared in the following way for analysis. To 500 μl of plasma in a 1.5 ml Eppendorf tube, 50 μl of 30% w/v aqueous perchloric acid was added with continuous mixing (Auto Vortex Mixer MT19, Chiltern Scientific) to precipitate the plasma proteins. The tubes were centrifuged for 10 min at 1400*g* and up to 30 μl of the clear supernatant was injected directly into the HPLC system by the WISP.

Validation of the plasma assay. The linearity and the precision for the plasma assay was determined by repeated analysis of plasma paracetamol standards, with concentrations ranging from 5 to 25 $\mu l\ ml^{-1}$. Samples were assayed as described with the modification that 50 μl of the internal standard solution was added, and analysed six times over a period of 2 days. The recovery of the assay was assessed using the method of standard addition and also by comparison with commercially available plasma paracetamol standards.

Calculation of results for the plasma assay. Concentrations of paracetamol and its metabolites in plasma were calculated in paracetamol equivalents from the peak area values. Standard curves were calculated by the least-squares regression analysis of peak area vs drug concentration. The reciprocal of the slope of the regression line is the factor by which the peak area is multiplied to give the paracetamol concentration in $\mu g\ ml^{-1}$. For the precision and linearity validation, calculations were done using both the peak area counts and also the peak area ratios (paracetamol peak area to internal standard peak area).

As mentioned for the urine assay, only paracetamol and not its metabolites was available in sufficient quantities use as standards for each batch of samples, resulting in the concentrations of the metabolites being calculated as paracetamol equivalents. The UV absorbance of the glucuronide and sulphate conjugates of paracetamol is not the same as paracetamol and correction factors of 0.835 for the glucuronide conjugate and 1.027 for the sulphate conjugates in plasma at 254 nm were applied. No correction factor was used for the cysteine and the mercapturate conjugates [22].

The assay method was used for the analysis of plasma and urine samples from six normal healthy volunteers (four males, two females, aged 23–53 years) following a therapeutic dose of 20 mg of paracetamol per kilogram body weight (Panadol Syrup 160 mg/5 ml, Winthrop Product Inc., USA). Venous blood samples were taken at 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 24 h after dosing. Urine was collected for 24 h in separate fractions with chloroform added as a preservative to prevent breakdown of the metabolites.

Results and Discussion

Results of the urine assay

A chromatogram for a urine sample spiked with paracetamol and its metabolites is shown in Fig. 1. The retention times of paracetamol glucuronide, paracetamol sulphate, paracetamol cysteine, unchanged paracetamol, internal standard AAP and paracetamol mercapturate conjugates were 3.7, 9.1, 9.9, 11.8, 23.0 and 28.7 min, respectively. The limit of detection for the assay was dependent on the presence of interfering peaks from endogenous material present in the urine. Generally, as little as 1–2 ng of paracetamol can be detected

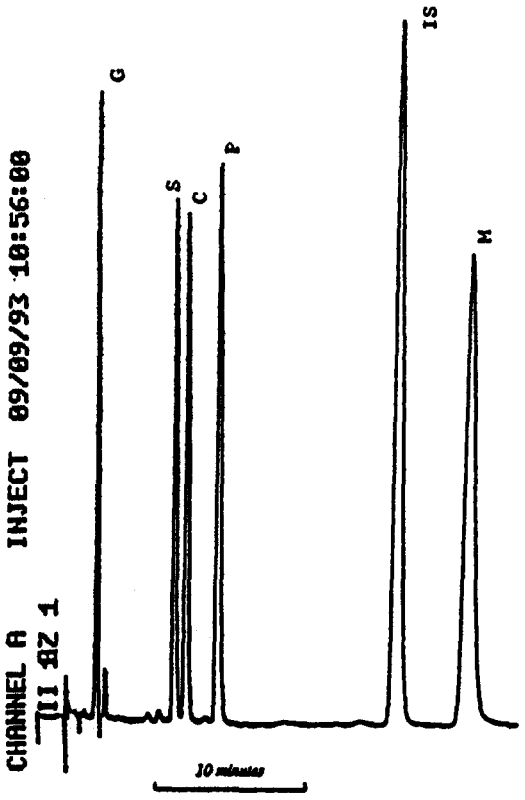

Figure 1
Chromatogram of paracetamol and its glucuronide (G), sulphate (S), cysteine (C) and mercapturate (M) metabolites in urine. Concentration: P, S, C = 10 μg ml^{-1}; G = 15 μg ml^{-1}; M = 30 μg ml^{-1}; IS = 27.4 μg ml^{-1}.

distinctly as a single peak, but this small amount is unlikely to be encountered in 24-h urine collections following therapeutic dosages as urinary collections of paracetamol are then measured in terms of milligrams. For the glucuronide, sulphate and cysteine metabolites, the detection limits were all below 5 ng, in paracetamol equivalents. The mercapturate metabolite is eluted last in this assay and the detection limit is higher but still below 10 ng.

Results of the validation of the urine assay are shown in Table 1. The relative standard deviation (RSD) was 0.2–1.7% using peak areas for calculation and 0.3–1.5% using peak area ratios. The plot of mean peak area and mean peak area ratio against concentration was linear and passed through the origin. There was excellent agreement between the results obtained by the two methods of calculation (Fig. 2).

The precision and linearity of the urine assay for the metabolites were also established. Serial dilutions of stock solutions of the glucuronide, sulphate, cysteine and mercapturate metabolites were made, and each sample was analysed five times. The results are shown in Table 2. The highest RSDs obtained were 1.2, 2.1, 2.4 and 16.3% for the glucuronide, sulphate, cysteine and mercapturate con-

Table 1
Precision of HPLC urine paracetamol assay (5–500 μg ml^{-1}) using peak area counts and peak area ratios

Paracetamol conc. (μg ml^{-1})	Peak area counts mean of four runs ± SD	R.S.D. (%)	Peak area ratios mean of four runs ± SD	R.S.D. (%)
5	64318 ± 901	1.4	0.191 ± 0.003	1.5
25	290559 ± 4011	1.4	0.860 ± 0.011	1.2
50	620752 ± 7083	1.1	1.841 ± 0.013	0.7
100	1165256 ± 8374	0.7	3.468 ± 0.012	0.3
250	2829170 ± 48601	1.7	8.327 ± 0.117	1.4
350	3908475 ± 6514	0.2	11.488 ± 0.074	0.6
500	5555135 ± 19319	0.3	16.335 ±0.213	1.3

Table 2
Details of the standard curves for urinary assay of paracetamol and its glucuronide, sulphate, cysteine and mercapturate metabolites (using peak area counts)

	Glucuronide	Sulphate	Cysteine	Paracetamol	Mercapturate
Slope	17548	10524	11926	11064	3620
Intercept	32574	4600	−26251	38861	−5277
Standard error of slope	24.1	15.9	7.7	57.8	10.8
Standard error of intercept	33177	57085	23744	26761	3579
Concentration range (μg ml^{-1})	5.7–1458	7.3–3750	3.1–3125	5–500	5.9–375
Number of standards	9	10	11	7	7
Correlation coefficient	0.999	0.999	0.999	0.999	0.999

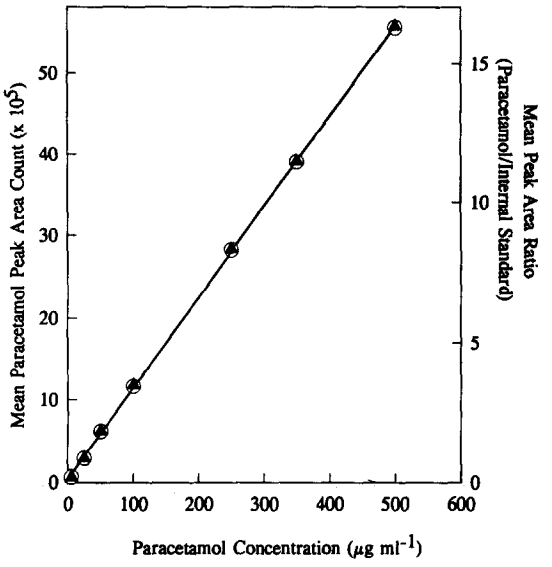

Figure 2
Linearity of HPLC urine assay of paracetamol using peak area counts (○) and peak area ratios (▲). Concentration: P, S, C = 10 μg ml^{-1}; G = 15 μg ml^{-1}; M = 30 μg ml^{-1}; IS = 27.4 μg ml^{-1}.

jugates, respectively. The peak areas all showed excellent linearity over the range of concentration studied.

Results of plasma assay

A chromatogram for a plasma sample spiked with paracetamol and its metabolites is shown in Fig. 3. The concentrations of paracetamol and its metabolites in the spiked sample are in the expected range following therapeutic dosage except for cysteine and mercapturate. The retention times of paracetamol glucuronide, paracetamol sulphate, paracetamol cysteine, unchanged paracetamol and paracetamol mercapturate were essentially the same as in the urine assay as the chromatographic conditions were almost identical. They were 3.7, 9.1, 9.9, 11.8 and 28.7 min, respectively. Paracetamol cysteine and paracetamol mercapturate could be detected by this HPLC system but they are in very low concentrations in plasma following therapeutic doses. A paracetamol cysteine peak could be seen in the majority of plasma samples following a therapeutic dose in our studies but a paracetamol mercapturate peak was never seen in any of our plasma samples. The absence of the mercapturate conjugate in the plasma of our study subjects is most likely due to its being present only in extremely low concentrations. Most previous published

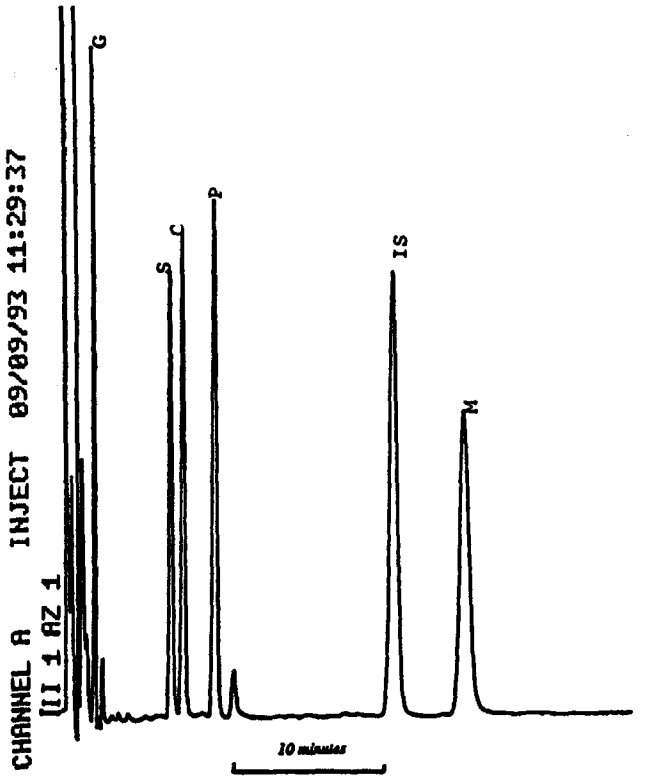

Figure 3
Chromatogram of paracetamol and its glucuronide (G), sulphate (S), cysteine (C) and mercapturate (M) metabolites in plasma.

studies only report the glucuronide and sulphate conjugates of paracetamol in plasma following therapeutic doses. In the present study, the plasma assay developed could detect the cysteine conjugate in most plasma samples. The non-detection of the mercapturate metabolite in *all* our plasma samples indicates that it could only be present in minute quantities in plasma relative to the concentrations of the cysteine conjugate as the detection limit of the latter differed from that of the mercapturate conjugate by less than 2-fold.

The detection limit of paracetamol was about 1–2 ng and there was no interference from other commonly used medications. The detection limits of the metabolites in plasma are similar to those in urine (i.e. below 5–10 ng).

Results of the validation of the linearity and precision of the plasma assay are shown in Table 3. The RSDs ranged from 0.1 to 3.3% using the peak area counts for calculation, while the calibration plots were linear and passed through the origin.

When using the peak area ratio (paracetamol peak area to internal standard peak area) for calculation, the variation in the peak areas of the internal standard had a larger percentage of error. The RSD in this case varied from 2.3 to 5.1% and reflected the relatively higher variation of the peak area counts of the internal standard of about 4%. Although the standard of accuracy and precision of modern laboratory pipettes are extremely good, the introduction of another variable, the dispensing of the internal standard in a volume of 50 μl, is still liable to produce a slight increase in the RSD of the calculated result, due to the degree of precision of both the pipette and the laboratory personnel.

Using the method of standard addition, the percentage of recovery was found to be consistently high, being very close to 100% (Table 4). Another set of recovery tests was done using commercially available plasma paracetamol standards for an ELISA method of paracetamol concentration measurement routinely employed for toxicological purposes by the Chemical Pathology Department of the Prince of Wales Hospital, Hong Kong. The concentrations of these standards estimated by the HPLC method were very close to their nominal value, as used in the ELISA assays, giving recoveries ranging from 94.9 to 96.3% over a range of 10–200 μg ml^{-1} (Table 5). It was thus deemed satisfactory to use the area counts for all calculations instead of the area ratio. The RSD of the area ratio (2.3–5.2%, median 3.3%) having been found to be higher than the RSD of the area counts (0.1–3.3%, median 0.3%). As precision, linearity and recovery were all above that required, it was decided not to use an internal standard. Thus standard curves calculated by the least-squares regression analysis of the peak area count vs drug concentration were used.

The linearity of the plasma assay for the metabolites was also established up to about 25 μg ml^{-1}. Greater concentrations are unlikely to be reached after a therapeutic dose of paracetamol. The coefficients of correlation are shown in Table 6. The linearity of the assays of all the metabolites and of paracetamol was found to be excellent.

The peak area counts obtained from paracetamol standards in plasma and in water were found to be different even if subjected to identical sample treatments. Peak area counts from paracetamol standards spiked into plasma were consistently lower than those spiked into water (data not shown). The reasons for this finding are unknown but it is speculated that a degree of plasma protein binding may be involved. However, to allow for this, all

Table 3
Precision of HPLC plasma paracetamol assay (5–25 μg ml^{-1}) using peak area counts and peak area ratios

Conc. (μg ml^{-1})	Peak area counts mean of six runs ± SD	R.S.D. (%)	Peak area ratios mean of six runs ± SD	R.S.D. (%)
5	542608 ± 17702	3.3	0.497 ± 0.025	5.1
10	1209332 ± 2694	0.2	0.970 ± 0.032	3.3
15	1804191 ± 6451	0.4	1.421 ± 0.046	3.3
20	2406085 ± 2322	0.1	1.885 ± 0.044	2.3
25	3056078 ± 7786	0.3	2.361 ± 0.123	5.2

Table 4
Recovery of HPLC plasma paracetamol assay using method of standard addition*

	Paracetamol area counts	Calculated paracetamol concentration ($\mu g\ ml^{-1}$)	Expected paracetamol concentration ($\mu g\ ml^{-1}$)	Recovery (%)
Baseline (B)	1357881	13.58	13.64	99.6
500 μl of 15 $\mu g\ ml^{-1}$ + 50 μl blank plasma	1347283	13.47		98.8
	1343479	13.44		98.5
	1332395	13.33		97.7
B + 1.25 μg/50 μl	1593451	15.91	15.91	100.0
	1580693	15.79		99.2
	1570506	15.68		98.6
	1561082	15.59		98.0
B + 2.5 μg/50 μl	1823175	18.18	18.18	100.0
	1809284	18.05		99.3
	1795566	17.91		98.5
	1783409	17.79		97.9
B + 5.0 μg/50 μl	2262295	22.53	22.73	99.1
	2244680	22.36		98.4
	2235913	22.27		98.0
	2230630	22.22		97.8

*Sample preparation: to 500 μl of a paracetamol plasma sample (15 $\mu g\ ml^{-1}$), 50 μl of plasma containing different amounts of paracetamol standard was added. Fifty microlitres of blank plasma was added to the baseline sample. Plasma protein precipitation was carried out in the normal manner.

Table 5
Recovery of HPLC plasma paracetamol assay using commercially available paracetamol standards

Sample	Peak area of paracetamol	Calculated paracetamol concentration ($\mu g\ ml^{-1}$)	Nominal paracetamol concentration ($\mu g\ ml^{-1}$)	Recovery (%)
A	0	0	0	—
A	0	0	0	—
A	0	0	0	—
B	964167	9.49	10.00	94.9
B	969433	9.54	10.00	95.4
B	962742	9.48	10.00	94.8
C	1948185	19.18	20.00	95.9
C	1946623	19.16	20.00	95.8
C	1936329	19.06	20.00	95.3
D	4908800	48.32	50.00	96.6
D	4859982	47.84	50.00	95.7
D	4864914	47.89	50.00	95.8
E	9774972	96.22	100.00	96.2
E	9825479	96.72	100.00	96.7
E	9783287	96.30	100.00	93.3
F	19638342	193.31	200.00	96.7
F	19402915	191.00	200.00	95.5
F	19388033	190.85	200.00	95.4

Table 6
Details of the standard curves for plasma assay of paracetamol and its glucuronide, sulphate, cysteine and mercapturate metabolites (using peak area counts)

	Glucuronide	Sulphate	Cysteine	Paracetamol	Mercapturate
Slope	40547	54266	68704	73278	49320
Intercept	10548	4152	−5538	39287	1466
Standard error of slope	869	816	1761	5360	826
Standard error of intercept	12826	12907	27841	90408	13011
Concentration range ($\mu g\ ml^{-1}$)	4.7–23.3	5.0–25.0	5.0–25.0	5.3–26.7	5.0–24.9
Number of standards	5	5	5	5	5
Correlation coefficient	0.999	0.999	0.999	0.992	0.999

Table 7
Urinary recoveries of paracetamol and its metabolites in 24-h collection in six healthy subjects following ingestion of a 20 mg kg^{-1} body weight dose

	Amount recovered in 24-h urine collection (mg)						Recovery
Subject	Gluc.	Sulp.	Cyst.	Para.	Merc.	Total	(% of dose)
1	730.89	271.59	26.91	34.22	26.20	1089.81	96.8
2	504.39	267.77	44.81	54.13	21.44	892.54	94.2
3	351.66	369.41	17.21	42.85	14.36	795.49	81.3
4	658.24	438.97	24.44	56.88	18.54	1197.07	99.6
5	641.83	459.45	57.39	84.47	58.36	1301.50	95.4
6	537.22	403.91	52.71	91.92	46.00	1131.76	98.2

reference standards to be run with plasma samples were prepared in paracetamol-free plasma.

The mean plasma concentration curves of paracetamol and its metabolites for the six healthy volunteers following a therapeutic dose of 20 mg kg^{-1} are shown in Fig. 4 and the urinary recoveries in Table 7.

Conclusions

The needs of clinical toxicology have been the main driving force behind the many published analytical methods available for the measurement of paracetamol concentrations in biological fluids. HPLC assays are particularly sensitive and accurate, and can measure therapeutic as well as overdose concentrations of paracetamol. However, in only a few published assays can the major metabolites of paracetamol (i.e. the glucuronide, sulphate, cysteine and mercapturate conjugates) be co-chromatographed by the same assay. The latter characteristics are necessary for full and

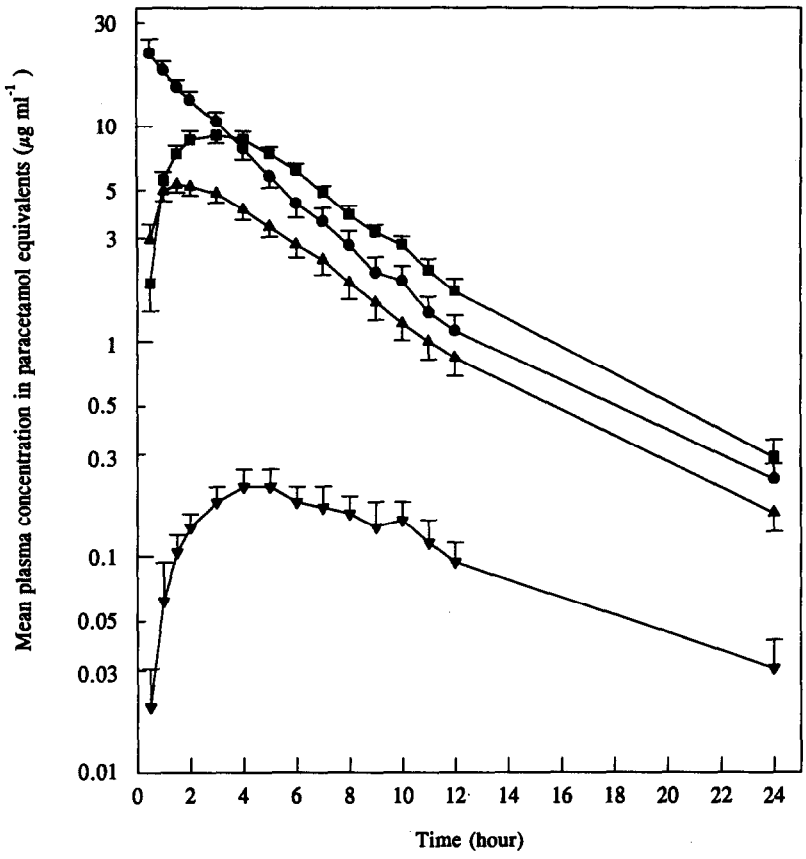

Figure 4
Mean plasma concentration (±SEM) of paracetamol (●) and its glucuronide (■), sulphate (▲) and cysteine (▼) metabolites in six healthy subjects following ingestion of a 20 mg kg^{-1} body weight dose.

efficient studies of the *in vivo* metabolism of paracetamol.

For the latter purpose, an HPLC method with excellent performance in precision, accuracy and linearity for the estimation of paracetamol and its metabolites is described. The same assay can be used to analyse both plasma samples and urine samples. Unlike previously published methods using UV detection, it can reliably detect the concentrations of the cysteine conjugate achieved in plasma following therapeutic paracetamol dosage. The method requires minimal sample preparation and has a relatively short run time. Validation tests showed that this assay method is reliable for both plasma and urine samples. It was used throughout for all our studies on the metabolism of paracetamol in healthy subjects and in patients with liver diseases including hepatocellular carcinoma. The plasma concentration data enables detailed pharmacokinetic modelling for paracetamol and its metabolites.

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[Received for review 8 March 1994; revised manuscript received 9 May 1994]