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# The quantification of paracetamol, paracetamol glucuronide and paracetamol sulphate in plasma and urine using a single high-performance liquid chromatography assay

L.S. Jensen, J. Valentine*, R.W. Milne, A.M. Evans

*Centre for Pharmaceutical Research, University of South Australia, Level 4, Reid Building, Frome Road, Adelaide, SA, Australia*

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**Abstract**

A range of analytical methods exist for the determination of paracetamol in biological fluids. However, to understand the fate of paracetamol and the effect of other drugs on its disposition in vivo, the major metabolites require quantification in urine and plasma. A method to simultaneously quantify paracetamol, paracetamol glucuronide (PG) and paracetamol sulphate (PS) in plasma and urine with superior sensitivity is therefore desired, especially if the volume of plasma available is low. A simple isocratic reverse phase high-performance liquid chromatography (HPLC) assay with spectrophotometric detection has been developed. The method, requiring only 100 μl of plasma and 50 μl of urine, utilizes a reversed-phase C18 column, a wavelength of 254 nm for detection and a mobile phase composed of potassium dihydrogen orthophosphate (0.1 M)–isopropanol–tetrahydrofuran (THF) (100:1.5:0.1, v/v/v) adjusted to pH 3.7 with phosphoric acid. The method is sensitive and linear in plasma within a concentration range from 0.4 to 200 μM for paracetamol, PG and PS. For PG and PS in urine, the method is sensitive and linear within a concentration range from 100 to 20,000 μM. Over these ranges, accuracy and precision were less than 12%. The assay has been used to measure concentrations of paracetamol and the two metabolites in plasma collected by finger-prick sampling and of the metabolites in urine from healthy volunteers administered a single oral dose of 1000 mg of paracetamol.


*Keywords:* HPLC; Paracetamol; Paracetamol glucuronide; Paracetamol sulphate; Plasma; Urine

## 1. Introduction

Paracetamol (acetaminophen) is commonly prescribed for the relief of mild to moderate pain and as an antipyretic. It is rapidly absorbed from the gastrointestinal tract and is primarily metabolized by conjugation with glucuronic and sulphuric acid to form paracetamol glucuronide (PG) and paracetamol sulphate (PS), respectively. These polar conjugates are subsequently cleared in the urine [1]. For studies on the disposition of paracetamol and the influence of other drugs on its disposition in humans, an assay that measures PG, PS and the unchanged drug is often required [2,3].

* Corresponding author. Tel.: +61-8-302-2463; fax: +61-8-302-1163.
*E-mail address:* jason.valentine@unisa.edu.au (J. Valentine).

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Historically, several different techniques have been used to measure paracetamol in biological fluids, including colorimetry, spectrophotometry, gas–liquid chromatography, thin layer chromatography and immunoassays. However, more recently, the preferred technique has been high-performance liquid chromatography (HPLC), due to the improved selectivity and sensitivity inherent in the technique [4–6]. Analytical methods have been developed that estimate the concentrations of the major metabolites of paracetamol by hydrolyzing the PG and PS back to paracetamol, or reporting concentrations of the metabolites as paracetamol equivalents [2,7]. Other assays quantify the major metabolites but they involve relatively complex liquid–liquid extraction techniques [8], gradient chromatography [9] or lack the sensitivity required for single-dose metabolism studies in humans [2,3]. While some assays report adequate sensitivity they lack the accuracy and precision required in single-dose studies, and therefore have not been applied [9]. This document reports the successful development and validation of a simple, selective and sensitive isocratic HPLC method for the simultaneous quantification of paracetamol in plasma and of PG and PS in both plasma and urine. A distinct advantage of this method is that it requires minimal sample volume (<100 μl) allowing finger-prick blood sampling during pharmacokinetic evaluation studies. Results of validation studies, including the accuracy, precision and sensitivity of the method for paracetamol, PG and PS are presented along with mean pharmacokinetic profiles of paracetamol, and the two metabolites in six healthy volunteers dosed with 1000 mg of paracetamol.

## 2. Materials and methods

Paracetamol and PG were purchased from Sigma (Castle Hill, NSW, Australia). PS was synthesized in-house following a previously published procedure [10] with modifications [11]. The identity of the PS was confirmed using NMR and the purity (79.1%) was calculated via hydrolysis of the material and comparison with authentic paracetamol. Potassium dihydrogen orthophosphate was obtained from Merck (Kilsyth, Vic., Australia). Isopropanol, tetrahydrofuran, 30% perchloric acid and acetic acid were obtained from BDH Laboratory Supplies (Poole, UK). Water was purified using a Milli-Q® water purification system. Analyte-free urine and plasma used for the preparation of calibration standards and quality control (QC) samples were obtained from volunteers and screened for interfering peaks prior to use.

### 2.1. Chromatographic equipment and conditions

The HPLC system consisted of a Hitachi 655A-11 pump, a SIL10ADvp auto injector (Shimadzu Corporation, Kyoto, Japan), a SPD-10Avp UV-Vis detector (Shimadzu Corporation) set at a wavelength of 254 nm and coupled to a CR-5A Chromatpac integrator (Shimadzu Corporation). A mobile phase consisting of potassium dihydrogen orthophosphate (0.1 M)–isopropanol–tetrahydrofuran (THF) (100:1.5:0.1, v/v/v, pH 3.7, adjusted with phosphoric acid) was circulated through a platinum EPS C18 analytical column (Alltech, Box Hill, Vic., Australia) with dimensions of 250 mm × 4.6 mm and a particle size of 5 μm, at a flow rate of 1 ml $min^{-1}$. Typical retention times were 3.7, 7.5 and 10.5 min for PG, PS and paracetamol, respectively. The total run times for the plasma and urine assays were 22 min.

### 2.2. Collection and storage of test samples

Blood samples were collected into labelled 250 μl blood collection tubes, centrifuged at 1800 × *g* for 5 min and the plasma layer frozen at −20 °C. Prior to analysis a 100 μl sample of the plasma was transferred into an Eppendorf tube and diluted with 100 μl of Milli-Q® water. Urine samples (50 μl) were frozen at −20 °C after collection. The urine was thawed prior to analysis, transferred to an Eppendorf tube and diluted with Milli-Q® water to give a total volume of 1 ml.

### 2.3. Plasma assay

#### 2.3.1. Preparation of standard, quality control and test samples

A stock solution was prepared containing equivalent concentrations of paracetamol, PG and PS (2000 μM). Appropriate dilutions were performed to prepare calibration standards of the three analytes at concentrations of 0.4, 1, 2, 4, 20, 40, 100 and 200 μM in 100 μl of drug-free plasma. The standards were diluted with Milli-Q® water to give a total volume of 200 μl. A

stock solution was prepared from a separate weighing of the three analytes, and was used to prepare quality control samples containing paracetamol (1.2, 32 and 160 μM) and the two metabolites (3.0, 32 and 160 μM). Aliquots of quality controls (100 μl) were diluted with Milli-Q® water to give a total volume of 200 μl. To process an analytical run the standard, quality control and test samples were treated in the same manner. The samples were thawed at room temperature and 10 μl of 30% perchloric acid added to each tube to precipitate proteins. The samples were vortexed for 5 s and centrifuged at 1800 × *g* for 10 min. The supernatant was transferred to the HPLC and 20 μl injected onto the column.

### 2.4. Urine assay

#### 2.4.1. Preparation of standard, quality control and test samples

A stock solution was prepared containing equivalent concentrations of paracetamol, PG and PS (200,000 mM). Appropriate dilutions were performed to prepare calibration standards of the three analytes at concentrations of 100, 200, 400, 1000, 2000, 4000, 10,000 and 20,000 μM in 50 μl of drug-free urine. The standards were diluted with Milli-Q® water to give a total volume of 1 ml. A stock solution was prepared from a separate weighing of the three analytes, and was used to prepare quality control samples containing the three analytes (240, 3200 and 16,000 μM). Aliquots of the quality control samples (50 μl) were diluted with Milli-Q® water to give a total volume of 1 ml. To process an analytical run the standard, quality control and test samples were treated in the same manner. All samples were thawed, centrifuged at 1800 × *g* for 10 min, an aliquot transferred to the HPLC, and 20 μl injected onto the column.

#### 2.4.2. Validation of the plasma and urine assay

Each analytical run consisted of the eight calibration standards, duplicate quality control samples at each of the three levels, a drug-free urine or plasma sample and test samples. Standards and quality controls were randomly positioned throughout each analytical run. Six analytical runs were conducted to validate the plasma assay and five for the urine assay. Calibration curves were constructed by regression analysis using peak heights and a weighting of $1/y$. Test and quality control samples were quantified from the regression equation generated from standard samples in the same analytical run.

The assay was used for the analysis of plasma and urine samples from six healthy volunteers administered a single 1000 mg dose of paracetamol as two 500 mg tablets (Panadol™). Blood samples (250 μl) were collected into Eppendorf tubes pre-dose and at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12 and 24 h post-dose using finger-prick sampling. Urine samples were collected pre-dose and from 0 to 12 and 12 to 24 h post-dose.

## 3. Results and discussion

### 3.1. Plasma assay validation

#### 3.1.1. Selectivity

Paracetamol and its two major metabolites were adequately separated from the endogenous peaks in drug-free plasma. Plasma from twelve individuals was found to be free of interfering peaks. Chromatograms of drug-free plasma with and without the three analytes are shown in Fig. 1.

#### 3.1.2. Linearity

Six validation runs were conducted on separate days and the results of the standard curves obtained for paracetamol and the two metabolites are detailed in Table 1. The %CV for the slopes of the calibration curves for paracetamol, PG and PS were 2.4, 3.3 and 3.9, respectively. Mean correlation coefficients for

Table 1
Details of standard curves for paracetamol and its two metabolites in plasma ($n = 6$)

| | Paracetamol | Paracetamol glucuronide | Paracetamol sulphate |
|---|---|---|---|
| Slope | 62.7 | 174 | 64.9 |
| Intercept | 11.6 | 174 | 7.6 |
| %CV of slope | 2.4 | 3.3 | 3.9 |
| Concentration range (μM) | 0.4–200 | 0.4–200 | 0.4–200 |
| Number of standards | 8 | 6 | 6 |
| Correlation coefficient ($r^2$) | 0.9999 | 0.9999 | 0.9996 |

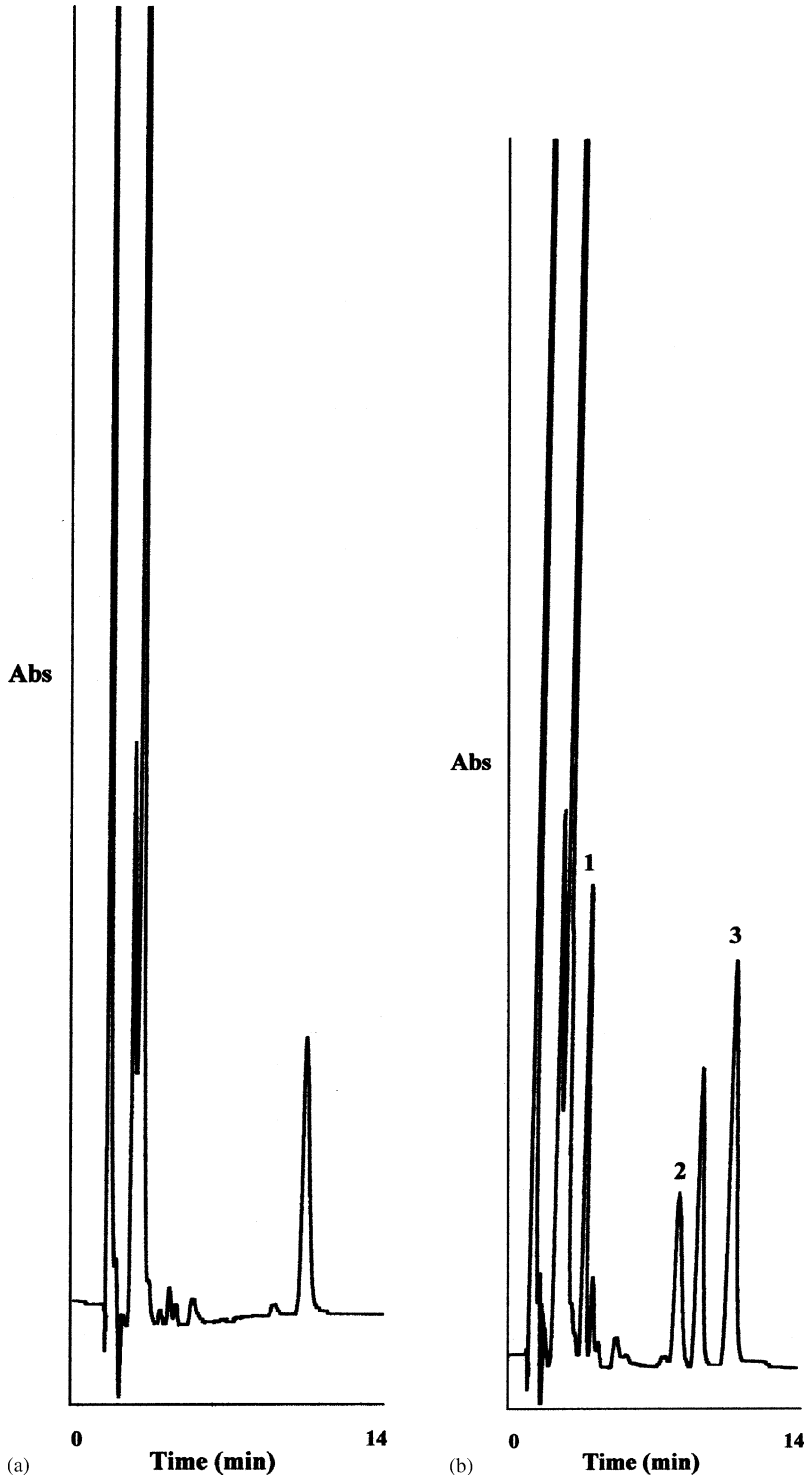


Fig. 1. (a) Drug-free plasma. (b) Plasma sample 6 h post-dose: (1) paracetamol glucuronide; (2) paracetamol sulphate; (3) paracetamol.

Table 2
Inter-day precision and accuracy of the standard and quality control samples for paracetamol and the two major metabolites in plasma

| Nominal concentration in plasma (μM) | Paracetamol | | | Paracetamol glucuronide | | | Paracetamol sulphate | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean observed concentration in (μM) | %CV | Percent difference from nominal concentration in (μM) | Mean observed concentration in (μM) | %CV | Percent difference from nominal concentration in (μM) | Mean observed concentration in (μM) | %CV | Percent difference from nominal concentration in (μM) |
| Standards ($n = 6$) | | | | | | | | | |
| 0.4 | 0.427 | 10.3 | 6.7 | 0.380 | 17.0 | −5.1 | 0.426 | 8.2 | 6.4 |
| 1 | 1.00 | 8.9 | 0.1 | 1.07 | 6.6 | 6.8 | 0.994 | 5.2 | −0.6 |
| 2 | 2.06 | 6.0 | 2.8 | 2.06 | 5.0 | 3.0 | 1.99 | 6.1 | −0.3 |
| 4 | 3.92 | 3.4 | −2.0 | 3.85 | 5.8 | −3.8 | 3.93 | 3.1 | −1.7 |
| 20 | 19.2 | 4.8 | −4.0 | 19.5 | 4.6 | −2.4 | 20.5 | 8.8 | 2.5 |
| 40 | 40.4 | 4.1 | 1.0 | 40.5 | 4.3 | 1.3 | 39.7 | 5.7 | −0.8 |
| 100 | 99.8 | 0.9 | −0.2 | 99.8 | 1.1 | −0.2 | 99.6 | 1.3 | −0.4 |
| 200 | 201 | 0.7 | 0.4 | 200 | 0.6 | 0.2 | 201 | 1.8 | 0.3 |
| Quality controls ($n = 10$) | | | | | | | | | |
| 1.2 | 1.29 | 10.8 | 7.1 | – | – | – | – | – | – |
| 3 | – | – | – | 2.95 | 6.7 | −1.6 | 3.16 | 9.7 | 5.4 |
| 32 | 32.0 | 4.1 | 0.1 | 32.2 | 3.9 | 0.7 | 33.3 | 5.8 | 4.1 |
| 160 | 158 | 5.0 | −1.4 | 157 | 4.9 | −1.7 | 162 | 3.9 | 1.1 |

Calculations use unrounded numbers.

paracetamol and PG were 0.9999. The mean correlation coefficient for PS was 0.9996.

### 3.1.3. Precision and accuracy of standard and quality control samples

The six validation runs were used to evaluate inter-day variability of standard and quality control samples. A summary of the inter-day results for paracetamol and the metabolites are shown in Table 2. The accuracy and precision of the assay at the limit of quantification (0.4 μM) were evaluated for the three analytes, and found to be acceptable. The %CV for paracetamol, PG and PS at the LOQ were 10.3, 17.0 and 8.2, respectively. The mean percent difference from the nominal concentration at the LOQ was 6.7, −5.1 and 6.4, respectively. The limit of detection (LOD) for all three analytes was 0.2 μM. At all other concentrations the accuracy and precision was within 10%. Results indicate that the method is linear, accurate and precise over the concentration ranges.

## 3.2. Urine assay validation

### 3.2.1. Selectivity

Paracetamol, PG and PS were adequately separated from each other and from endogenous peaks in the drug-free urine. Urine from twelve individuals was found to be free of interfering peaks. Chromatograms of drug-free urine with and without the three analytes are shown in Fig. 2.

Table 3
Details of standard curves for the two major metabolites of paracetamol in urine ($n = 5$)

| | Paracetamol glucuronide | Paracetamol sulphate |
|---|---|---|
| Slope | 10.3 | 4.90 |
| Intercept | 138 | 29.9 |
| %CV of slope | 6.2 | 6.6 |
| Concentration range (μM) | 100–20000 | 100–20000 |
| Number of standards | 8 | 8 |
| Correlation coefficient ($r^2$) | 0.9998 | 0.9998 |

### 3.2.2. Linearity

Five validation runs were conducted on separate days and the results of the standard curves obtained for the two metabolites are provided in Table 3. The standard curves showed good reproducibility for PG and PS with a %CV of 6.2 and 6.6 for the slope, respectively. The calibration curves showed good linearity over the concentration range tested, with mean correlation coefficients of 0.9998 for both metabolites.

Table 4
Inter-day precision and accuracy of the standard and quality control samples for the two major metabolites of paracetamol in urine

| Nominal concentration in urine (μM) | Paracetamol glucuronide | | | Paracetamol sulphate | | |
|---|---|---|---|---|---|---|
| | Mean observed concentration (μM) | %CV | Percent difference from nominal concentration (μM) | Mean observed concentration (μM) | %CV | Percent difference from nominal concentration (μM) |
| Standards ($n = 5$) | | | | | | |
| 100 | 102 | 6.8 | 1.7 | 106 | 7.2 | 6.4 |
| 200 | 201 | 2.9 | 0.4 | 201 | 1.7 | 0.6 |
| 400 | 406 | 4.3 | 1.5 | 405 | 4.2 | 1.2 |
| 1000 | 979 | 3.1 | −2.1 | 961 | 3.2 | −3.9 |
| 2000 | 1988 | 3.4 | −0.6 | 1963 | 3.0 | −1.8 |
| 4000 | 3991 | 1.4 | −1.2 | 3948 | 1.8 | −1.3 |
| 10000 | 10009 | 1.7 | 0.1 | 9935 | 1.7 | −0.7 |
| 20000 | 20033 | 0.9 | 0.2 | 20194 | 0.8 | 1.0 |
| Quality controls ($n = 10$) | | | | | | |
| 240 | 213 | 4.1 | −11.1 | 222 | 4.1 | −7.5 |
| 3200 | 2896 | 2.6 | 2.6 | 2937 | 3.1 | −8.2 |
| 16000 | 15992 | 2.2 | 2.2 | 16019 | 2.7 | 0.1 |

Calculations use unrounded numbers.

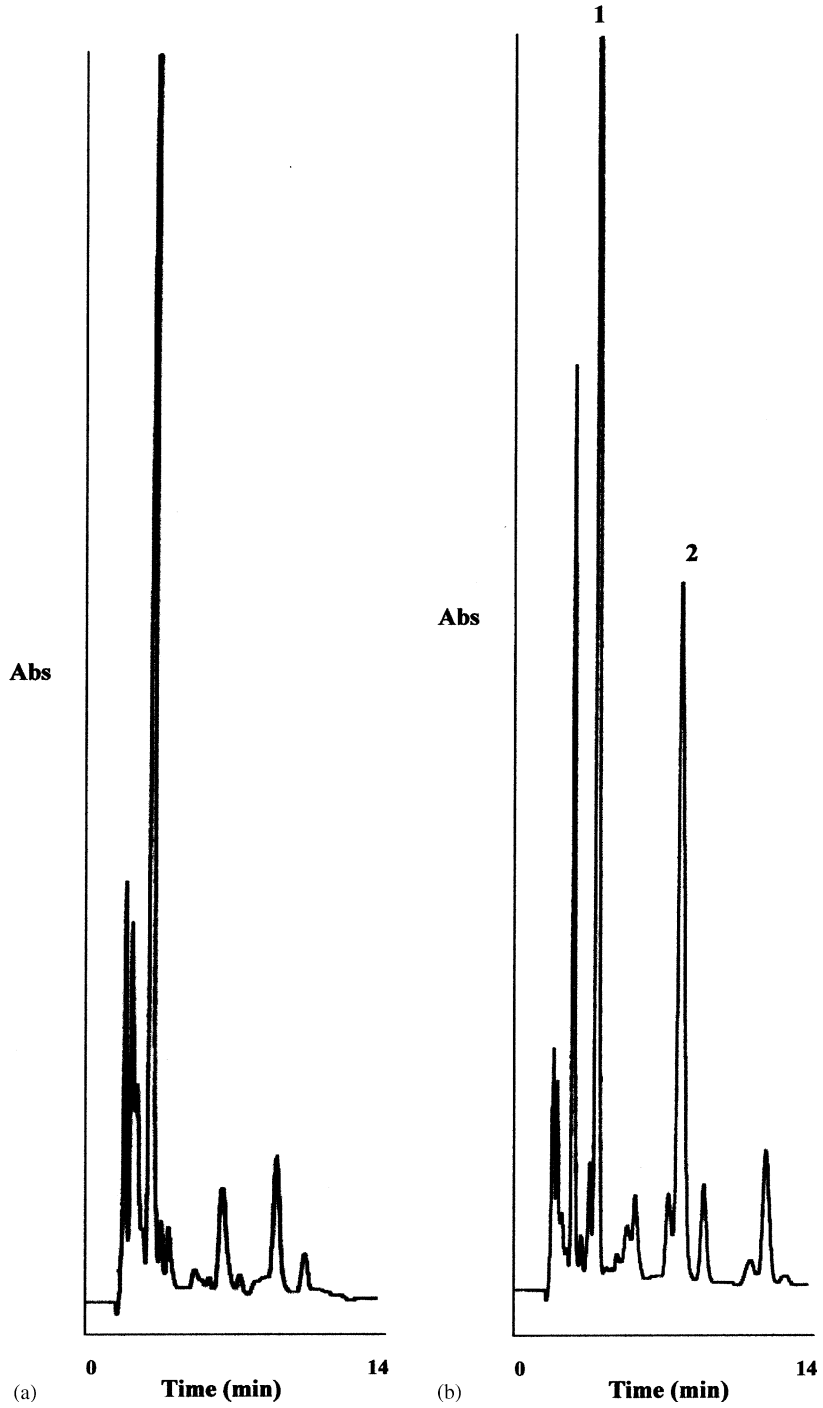


Fig. 2. (a) Drug-free urine. (b) Urine sample 0–12 h post-dose: (1) paracetamol glucuronide; (2) paracetamol sulphate.

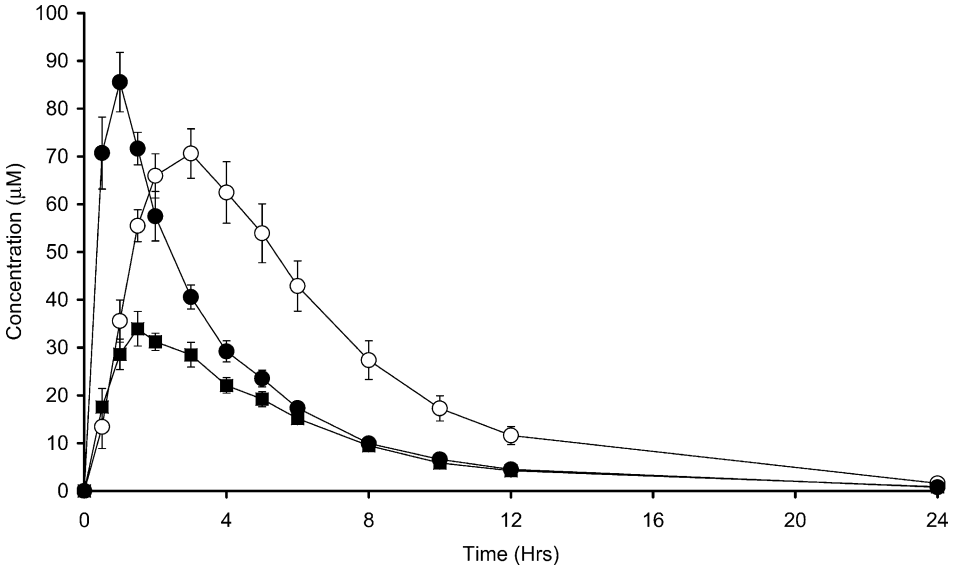


Fig. 3. Mean (±S.E.) plasma concentrations curves for paracetamol and its major metabolites for six healthy volunteers after a single 1000 mg dose: (●) paracetamol; (○) paracetamol glucuronide; (■) paracetamol sulphate.

### *3.2.3. Precision and accuracy of standard and quality control samples*

Five validation runs were conducted on separate days to evaluate the inter-day variability of standard and quality control samples and a summary of the results for the two metabolites is shown in Table 4. The accuracy and precision of the assay at the limit of quantification (100 μM) were evaluated for the major metabolites and were found to be acceptable. The %CV for PG and PS at the LOQ were 6.8 and 7.2,

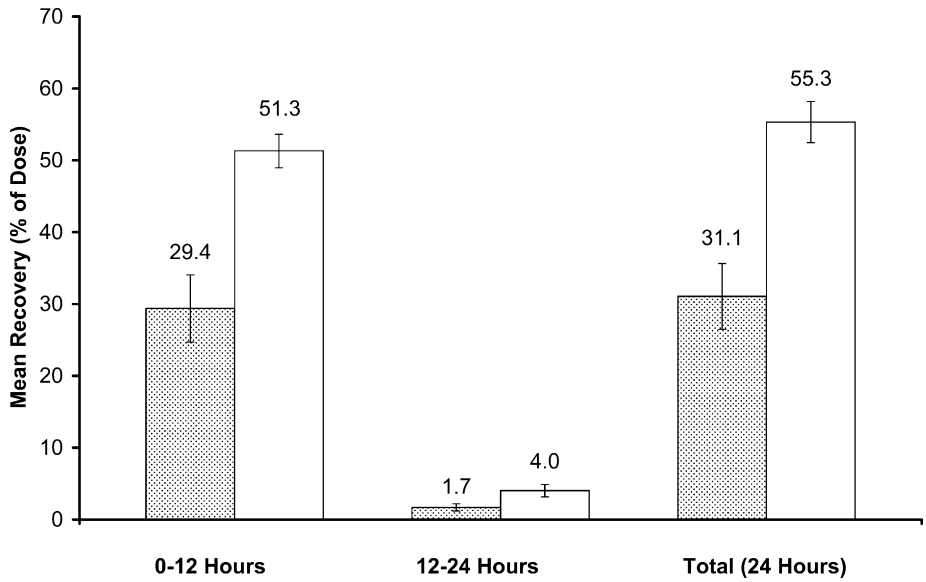


Fig. 4. Mean (±S.E.) urinary excretion for the major metabolites of paracetamol for six healthy volunteers after a 1000 mg dose: (shaded) paracetamol sulphate; (unshaded) paracetamol glucuronide.

respectively. The mean percent difference from the nominal concentration at the LOQ was 1.7 and 6.4, respectively. The results indicate that the method is linear, accurate and precise over the concentration range studied (100–20,000 μM).

### *3.3. Application of the method for measuring paracetamol and its major metabolites*

The mean concentrations in plasma versus time curves for paracetamol and its two major metabolites for six healthy volunteers after a single 1000 mg dose are shown in Fig. 3, and are similar to concentrations reported in studies conducted elsewhere [2,3]. As can be seen from the concentration profiles in plasma, the method is sufficiently sensitive for the analysis of paracetamol, PG and PS in 100 μl samples collected for up to 24 h after a single dose of paracetamol. The mean percent of dose excreted in urine for the two major paracetamol metabolites for the six subjects was 55.3 and 31.1 for PG and PS, respectively. The urinary excretion of PG and PS is summarized in Fig. 4.

## 4. Conclusion

Studies involving the disposition of paracetamol in humans frequently require a sensitive, accurate and precise assay that is capable of quantifying paracetamol and its major metabolites within the same analytical run in both plasma and urine. This especially applies to studies examining the influence of other drugs on the disposition of paracetamol. Very few analytical methods are available that measure the two major metabolites of paracetamol directly and even fewer have the accuracy, precision and sensitivity to analyse samples from single-dose pharmacokinetic studies. The simple assay described here is accurate and precise and allows measurement of the concentrations of paracetamol and its two major metabolites simultaneously in plasma and in urine. An additional advantage of the method is that concentrations in low volumes of plasma obtained from a finger-prick may be measured with sufficient sensitivity to characterize the concentrations in plasma versus time profiles over almost two orders of magnitude.

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