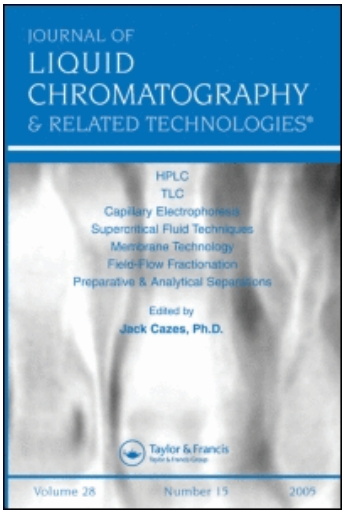

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A VALIDATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF PARACETAMOL AND ITS MAJOR METABOLITES IN URINE

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ABSTRACT

A reversed-phase high-performance liquid chromatographic (RP-HPLC) method employing UV-detection (248 nm), 5 μm Resolve C18 as stationary phase (in a 8 x 100 mm Waters Radial-Pak cartridge) and 0.1 M potassium phosphate monobasic/methanol/glacial acetic acid (95:4:1, v/v/v) as mobile phase was developed for the rapid and simultaneous determination of paracetamol and its major metabolites (its glucuronide and sulphate conjugates) in urine. The method requires only minimal sample preparation and chromatographic run time is only 6 min. For determination of paracetamol, the precision (inter-assay RSD ranged from 1.60-0.66% between 5 and

100 μg mL^{-1}) and limits of detection (2 ng mL^{-1}) were satisfactory, as were these parameters for determination of the major metabolites. In the course of studies on paracetamol bioavailability and metabolism, over 1500 samples have been assayed using this method. Herein we report its use for monitoring of the levels of paracetamol and its major metabolites in the urine of fifteen normal healthy volunteers given a single oral dose of 500 mg.

INTRODUCTION

Paracetamol or acetaminophen (N-acetyl-*p*-aminophenol) is a mild analgesic-antipyretic used in the treatment of somatic pain (e.g. myalgia, arthralgia, neuralgia), often where aspirin (acetylsalicylic acid) is contraindicated (for example, in cases where there is gastrointestinal intolerance, hyperuricaemia, gastritis, asthma or von Willebrand disease) or when the analgesic is to be administered in conjunction with other drugs, such as non-steroidal inflammatory agents or oral anticoagulants [1].

Following oral administration of a therapeutic dose of paracetamol, it is rapidly absorbed from the gastrointestinal tract. Peak plasma levels are reached within 30 to 120 min, and the therapeutic half life is about 3 h, the bulk (ca. 98%) of the drug being metabolized in the hepatic system, primarily to its glucuronide and sulphate conjugates [2-4]. The incidence of the first pass effect on drug bioavailability depends on the dose, dosage interval and route of administration [5-7].

Many analytical techniques have been used to determine paracetamol in biological fluids, among them colorimetry [8,9], gas-liquid chromatography [10-15],

thin layer chromatography [16], high performance liquid chromatography [17-22] and immuno-assay techniques [23,24]. Nonetheless, since these assays were largely developed for use in clinical toxicology, they are not necessarily suitable for pharmacokinetic studies, which require precise measurement of very low levels of paracetamol and its metabolites and often involve analysis of large numbers of samples in short periods [25-28].

In this work we aimed to develop a paracetamol assay suitable for use in studies of its bioavailability and metabolism. In order to minimize sample preparation, a highly specific technique was called for. The technique chosen was HPLC with UV detection, which as well as specificity offered high sensitivity, and the possibility of semi-automation. The chromatographic run time was minimized by employing a Waters Radial-Pak cartridge and optimizing chromatographic efficiency, selectivity and resolution. The assay developed allows simultaneous determination of paracetamol and its major metabolites (its glucuronide and sulphate conjugates) in urine. The limits of detection and precision of the assay are reported, and also a specimen set of data for paracetamol levels in the urine of fifteen normal healthy volunteers over a 12 h period.

EXPERIMENTAL

Materials

Paracetamol was obtained from C. Barcia (Barcelona, Spain). Paracetamol sulphate and paracetamol glucuronide

were synthetized according to the methods of Burkhardt [30] and Shibasaki [31], respectively, in the Pharmaceutical Chemistry Laboratory, Pharmacy Faculty, University of Santiago de Compostela (Spain). HPLC grade methanol and glacial acetic acid were obtained from E. Merck (Darmstadt, Germany). Analytical grade monobasic potassium phosphate was obtained from Probus (Barcelona, Spain). Water for HPLC was purified using the Milli Q Water Purification System (Millipore, Bedford, MA, USA).

Sample collection

Following administration of a 500 mg dose of paracetamol, urine samples were collected after set time intervals. Urine volume was recorded and a 20 mL aliquot was stored at -20°C (at this temperature paracetamol and its conjugates are stable in plasma and urine) [32]. A urine blank, i.e. urine free of paracetamol and its metabolites, was also procured.

Preparation of standards and test samples

Stock solutions of 1 mg mL^{-1} paracetamol, paracetamol glucuronide and paracetamol sulphate in deionized water were prepared. Aliquots of each solution were then diluted with the urine blank to give standards having analyte concentrations of 5, 10, 15, 20, 25, 50 and 100 $\mu g\ mL^{-1}$.

The test samples were prepared by diluting each thawed 20 mL urine sample (including the urine blank) with a suitable volume of deionized water and filtering

this solution through a 0.45 μm hydrophilic membrane (Millex HV filter unit, Millipore, Bedford, USA). A sample of the filtrate was transferred to an injection in preparation for chromatography.

Chromatography

The HPLC system comprised a Waters 510 pump, a Waters Intelligent Sample Processor 712 automatic injection module, and a Waters Guard-Pak precolumn and Radial-Pak cartridge (8 mm i.d. x 100 mm) - the latter housed in a Waters Radial Compression Module (RCM8x10) - both packed with 5 m particle-size Resolve C18. The pressure of the system was maintained at 1200 p.s.i. UV-detection was by a Waters Lambda Max 490 (set at 248 nm) and data handling by a Waters 745 B Data Module. The mobile phase was 0.1 M potassium phosphate monobasic/methanol/glacial acetic acid (95:4:1, v/v/v), degassed by sonication; the flow rate was 2.5 mL^{-1} min. In all cases, 20 μL of sample/standard was injected onto the column.

Calibration

Calibration curves for each analyte were constructed by least-squares regression of peak area (mean of three replicates) on analyte concentration in the standard solutions. The peak areas for the test samples were converted to concentrations (in μg mL^{-1}) by multiplying them by the reciprocal slope of the corresponding calibration curve.

Precision

For evaluation of the precision, the paracetamol and the paracetamol glucuronide and sulphate standards were assayed as described four times and the relative standard deviation (RSD) for each of the seven concentrations (5 to 100 $\mu g\ mL^{-1}$) was calculated.

Application

Fifteen normal healthy volunteers were given a single oral dose of 500 mg paracetamol. Urine samples were collected after 1, 2, 3, 4, 5, 6, 8, 10 and 12 h and processed and assayed (three replicates) as described.

RESULTS AND DISCUSSION

Table 1 shows the parameters for the calibration curves. All three were linear with correlation coefficients > 0.998 over the range of concentrations studied.

Typical chromatograms for a diluted, filtered urine sample and the correspondingly processed urine blank are shown in Figs. 1(a) and 1(b) respectively. Figure 1(a) corresponds to urine obtained from a subject 3 h after a single oral dose of 500 mg of paracetamol. The overall sample run time was 6 min, and the retention times for paracetamol glucuronide, paracetamol sulphate and unchanged paracetamol were 2.15, 3.48 and 4.47 min, respectively. Fig. 1(b), showing the chromatogram of the urine blank, confirms that there were no signals due to

TABLE 1

Parameters of the Calibration Curves for the Determination of Paracetamol and its Glucuronide and Sulphate Metabolites in Urine (using peak area counts).

	Glucuronide	Sulphate	Paracetamol
Slope	1539543	1602243	124298
Intercept	6343569	2310349	54499
RSD of slope (%)	1.67	3.29	1.10
Conc. range (μg mL^{-1})	5-100	5-100	5-100
Number of standards	7	7	7
Correlation coeficient	0.9993	0.9987	0.9997

endogenous compounds at the retention times of the analytes. When endogenous compounds in the urine gave peaks close to these retention times, limits of detection were slightly affected. Notwithstanding, as little as 2 ng mL^{-1} of paracetamol and 5 ng mL^{-1} of its glucuronide and sulphate conjugates (in paracetamol equivalents) could be detected as a distinct peak, which is more than satisfactory since, even 12 h after therapeutic doses, urine concentrations of paracetamol are generally measured in terms of milligrams.

The precision is shown for paracetamol in Table 2. The relative standard deviation ranged from 1.60-0.66% between 5 and 100 μg mL^{-1} paracetamol, indicating that precision was satisfactory in the concentration range studied. For paracetamol glucuronide and sulphate, the RSDs ranged from 0.4-1.5% and 0.7-2.4%, respectively, in the same concentration range.

The method described here has been successfully used to assay paracetamol in over 1,500 urine samples in the course of studies on paracetamol bioavailability and

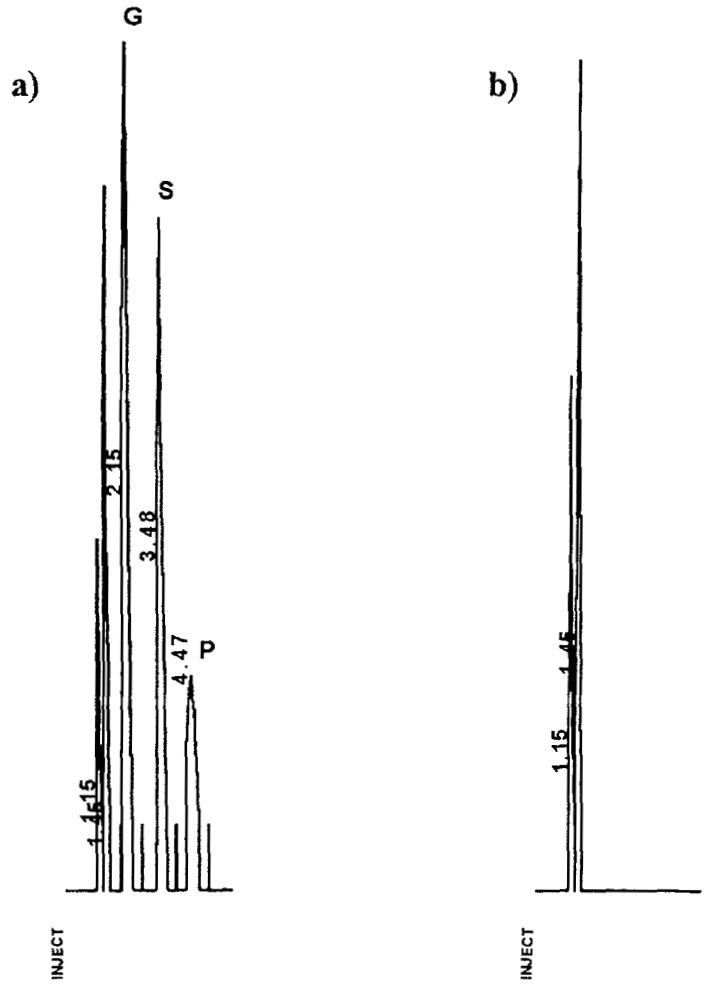

FIGURE 1. (a) Chromatogram of paracetamol (P) and its glucuronide (G) and sulphate (S) metabolites in urine. Concentrations P = 3.04 μg mL^{-1}; S = 27.72 μg mL^{-1}; G = 49.33 μg mL^{-1}. (b) Chromatogram of a processed urine blank.

TABLE 2

Precision (RSD) of the HPLC Assay for Paracetamol (5-100 $\mu g\ mL^{-1}$) in Urine (using peak area counts).

Paracetamol conc. ($\mu g\ mL^{-1}$)	Peak area counts. Mean of four runs ± SD	R.S.D (%)
5	640302±10250	1.60
10	1369182±19237	1.40
15	1916115±23723	1.23
20	2435508±32270	1.32
25	3133128±45283	1.44
50	6424224±54275	0.84
100	12430191±83242	0.66

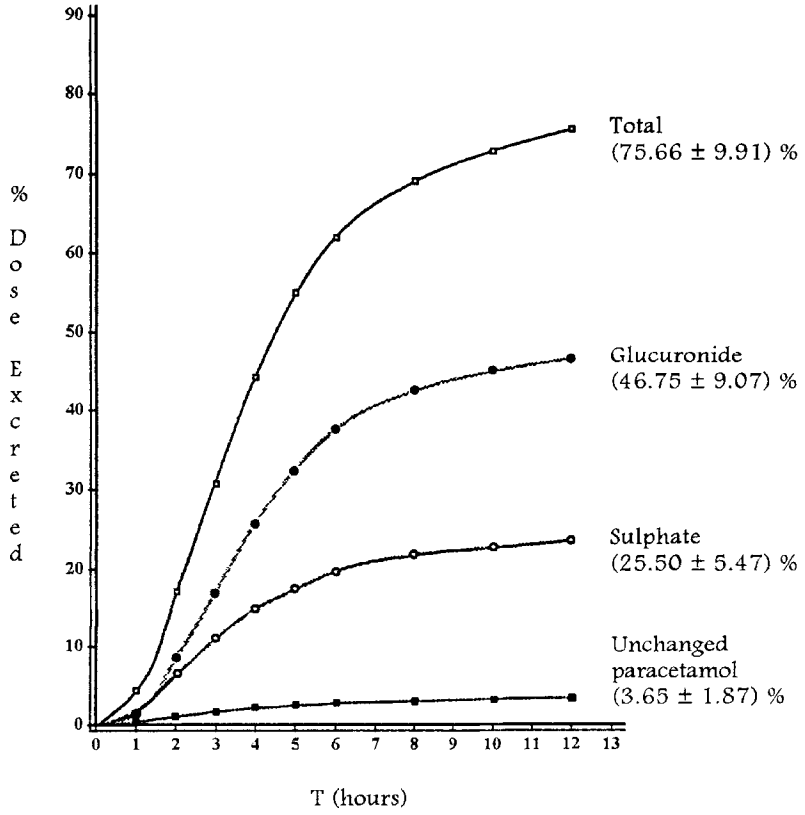

FIGURE 2. Cumulative curves of % dose excreted *vs* time for unchanged paracetamol, its glucuronide and sulphate conjugates, and the sum of these, after oral administration of a 500 mg dose to fifteen normal healthy volunteers.

metabolism [29]. Figure 2 shows the urinary profiles obtained for paracetamol and its glucuronide and sulphate conjugates in the short study described here. After 12 h, 3.65 ± 1.87% of the ingested paracetamol was excreted unchanged, and 25.50 ± 5.47% and 46.75 ± 9.07% was excreted as the sulphate and glucuronide conjugates, respectively (total, 75.66 ± 9.91%). These results are in keeping with those of HPLC assays for paracetamol employing conventional columns [17, 19, 21, 33].

CONCLUSIONS

An HPLC method was developed for the determination of paracetamol and its major metabolites, its glucuronide and sulphate conjugates, in urine. This method requires only minimal sample preparation and has a short chromatographic run time. The calibration curves are highly linear, and precision and limits of detection are satisfactory. This rapid method is especially suited to pharmacokinetic studies involving analysis of large numbers of samples, and has already been used to assay over 1500 samples in the course of studies on paracetamol bioavailability and metabolism.

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