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AUTOMATED LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF PARACETAMOL AND SIX METABOLITES IN HUMAN URINE

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SUMMARY

An automated liquid chromatographic method, with a coefficient of variation for total imprecision of less than 4%, has been developed for the quantitative determination of paracetamol, paracetamol-4-glucuronide, paracetamol-4-sulphate, paracetamol-3-cysteine, paracetamol-3-mercapturate, 3-hydroxyparacetamol-3-sulphate and 3-methoxyparacetamol-4-sulphate in urine samples. The gradient elution system was based on 0.067 *M* phosphate buffer (pH 2.0) and acetonitrile on an octadecylsilica column. The on-column detection limit using an ultraviolet detector at 254 nm for each of the compounds using 3-hydroxyacetanilide as internal standard was of the order of 10–50 ng from urine and 2–10 ng from water. Application of the method to 24-h urine samples from subjects who had received a therapeutic dose of the drug confirmed the findings of previous studies for the importance of the glucuronide, sulphate, mercapturate and cysteine conjugates. 3-Hydroxyparacetamol-3-sulphate was shown to be present in the urine of all volunteers and to account for up to 5% of the dose. 3-Methoxyparacetamol-4-sulphate was not detected in any urine samples and if present as a metabolite must account for less than 0.1% of the dose.

INTRODUCTION

Paracetamol (acetaminophen) is a readily available and widely used mild analgesic. Its common misuse in overdose situations and the concomitant production of irreversible liver damage has made the study of paracetamol metabolism of toxicological importance. Recent attention has focused on the use of H_2-receptor antagonists to minimise paracetamol-induced hepatotoxicity [1].

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In man, the two main urinary metabolites of paracetamol are the glucuronide and sulphate conjugates [2, 3]. In total, these two conjugates account for approximately 80% of the dose [3]. Minor metabolites which have been identified include paracetamol-3-cysteine and paracetamol-3-mercapturate, which arise from the reaction of a reactive intermediate with glutathione, 3-hydroxyparacetamol-3-sulphate, 3-methoxyparacetamol-4-sulphate and 3-methoxyparacetamol-4-glucuronide [2]. In addition 3-methylthioparacetamol has been identified as a possible metabolite [4]. The relative proportions of all of these metabolites appear to be different in therapeutic and overdose situations [2].

In view of the toxicological importance of paracetamol metabolism as well as the common use of the drug as model for Phase 2 metabolism, it is not surprising that many analytical methods have been reported for the identification of paracetamol and its metabolites in both human and animal studies. The most recent of these methods [5–16] have been based upon high-performance liquid chromatographic (HPLC) procedures using UV or electrochemical detectors and in some cases using ion-pairing agents [9, 10]. The majority of these previous studies, however, have only quantified paracetamol and its glucuronide, sulphate, cysteine and mercapturate conjugates; only Mrochek et al. [5] and Hamilton and Kissinger [12] have attempted to study 3-hydroxyparacetamol and the 3-methoxyparacetamol conjugates and in both cases they failed to quantify paracetamol-3-mercapturate. The method of Mrochek et al. was a very lengthy (45 h) ion-exchange procedure whilst the method of Hamilton and Kissinger used a reversed-phase isocratic system with an electrochemical detector. Wilson et al. [14] reported a method which used a combination of UV and electrochemical detection which separated paracetamol, its glucuronide, sulphate, cysteine and mercapturate conjugates together with unconjugated forms of the 3-hydroxyparacetamol, 3-methoxyparacetamol and 3-methylthioparacetamol metabolites but the conjugates of these metabolites were not studied directly. A criticism of many of these published methods is that they have given minimal quality control data to validate the analytical procedure.

The present paper reports a fully automated, quantitative method for determining paracetamol, its sulphate, glucuronide, cysteine and mercapturate conjugates, 3-hydroxyparacetamol-3-sulphate and the 3-methoxyparacetamol-4-sulphate conjugates in urine. Synthetic samples of 3-methoxyparacetamol-4-glucuronide and conjugates of 3-methylthioparacetamol were not available and were not included in the study.

EXPERIMENTAL

Chemicals

Paracetamol was obtained from Sigma (Poole, U.K.) and paracetamol-4-sulphate, paracetamol-4-glucuronide, paracetamol-3-cysteine, paracetamol-3-mercapturate, 3-hydroxyparacetamol-3-sulphate and 3-methoxyparacetamol-4-sulphate were all kindly donated by Dr. R.S. Andrews (Sterling Winthrop, Alnwick, U.K.). 3-Hydroxyacetanilide was used as internal standard.

Apparatus

A Perkin-Elmer Series 3B dual-pump liquid chromatograph fitted with a solvent programmer, Rheodyne Model 7105 injector with a 20-μl loop and Perkin-Elmer LC75 detector set at 254 nm was used in conjunction with a Perkin-Elmer 3B autosampler. A 25 mm $\times$ 4.6 mm Spherisorb ODS (5 μm particle size) column (Hichrom) was maintained at 30°C using a heater block (Jones Chromatography). A 50 mm $\times$ 4.6 mm guard column packed with 5-μm Spherisorb ODS was incorporated into the system.

The mobile phase consisted of an acetonitrile–phosphate buffer gradient. Acetonitrile (Rathbone Chemicals, Walkerburn, U.K.) and 0.067 *M* phosphate buffer (pH 2.0) were vacuum-degassed before use. Solvent A: phosphate buffer (pH 2.0); solvent B: phosphate buffer (pH 2.0)–acetonitrile (10:1, v/v). The solvent programme was 0–25 min solvent A–solvent B (99.9:0.1) and 25–52 min linear gradient from 0.1 to 70% solvent B; the flow-rate was 2.0 ml/min.

Sample preparation and calibration curves

Samples of test urine (2.0 ml) were diluted with distilled, deionised water (18.0 ml) and 3-hydroxyacetanilide (2 mg/ml; 200 μl) was added as internal standard. The solution was mixed for 30 s with a vortex-type mixer. A 300-μl sample was transferred in duplicate to autosampler tubes for analysis.

Calibration curves were prepared by adding known amounts of paracetamol, its six metabolites and internal standard to distilled water or blank urine. The analysis procedure was identical to that for test samples. All calibration curves were fitted by computer-assisted regression analysis and the correlation coefficients were greater than 0.997. The curves were linear over the concentration range studied [paracetamol up to 14 mmol/l (2.114 mg/ml), paracetamol-4-glucuronide up to 9 mmol/l (3.312 mg/ml); paracetamol-4-sulphate up to 11 mmol/l (3.157 mg/ml monopotassium salt); paracetamol-3-cysteine up to 8 mmol/l (2.16 mg/ml); paracetamol-3-mercapturate up to 7 mmol/l (2.184 mg/ml); 3-hydroxyparacetamol-3-sulphate up to 5 mmol/l (1.725 mg/ml dipotassium salt); 3-methoxyparacetamol-4-sulphate up to 3 mmol/l (0.96 mg/ml monopotassium salt)] and passed through the origin. The recovery and coefficient of variation of all compounds from blank urine were the same as those from distilled water. Standards were therefore routinely assayed from distilled water.

Analysis procedure and quality control

For the analysis of test urine samples a quality control urine and four standard aqueous solutions containing paracetamol and its six metabolites over the calibration range (one at either end of the range and two in the centre) were run at the beginning and end of each batch of samples. All test samples were analysed in duplicate. The control urine consisted of a test urine sample from a male volunteer, who had received a 1-g dose of the drug and who gave a 24-h urine volume of approximately 400 ml. It was aliquoted into 5-ml samples and kept at −20°C until required. It was analysed on 101 occasions, each one in duplicate. On the first 36 occasions (April to July, 1984) a column was used which resulted in the elution of paracetamol-4-sulphate before paracetamol-4-glucuronide. On the next

TABLE I

TOTAL AND WITHIN-BATCH DATA FOR THE ESTIMATION OF PARACETAMOL AND FIVE METABOLITES IN A QUALITY CONTROL TEST URINE ON 101 OCCASIONS

The analysis was carried out on a Spherisorb ODS column (Hichrom) using a phosphate buffer (pH 2.0) with an acetonitrile linear gradient. S.D. = standard deviation; C.V. = coefficient of variation.

Analyte	Period★	Mean (mmol/l)	Within-batch imprecision		Total imprecision		Total imprecision★★	
			S.D.	C.V. (%)	S.D.	C.V. (%)	S.D.	C.V. (%)
Paracetamol	A	0.479	0.0075	1.58	0.0087	1.82	0.0075	1.56
	B	0.486	0.0033	0.68	0.0053	1.10		
Paracetamol-4-sulphate	A	0.492	0.0094	1.92	0.0172	3.49	0.0131	2.69
	B	0.483	0.0062	1.28	0.0089	1.85		
Paracetamol-4-glucuronide	A	0.590	0.0063	1.07	0.0075	1.29	0.0063	1.08
	B	0.588	0.0044	0.75	0.0055	0.94		
Paracetamol-3-cysteine	A	9.998	0.1086	1.89	0.2494	2.49	0.2733	2.68
	B	10.336	0.1194	1.59	0.2026	1.96		
Paracetamol-3-mercapturate	A	5.352	0.1093	2.04	0.1719	3.21	0.2117	4.12
	B	5.016	0.0632	1.26	0.1146	2.29		
3-Hydroxyparacetamol-3-sulphate	A	0.450	0.0079	1.76	0.0155	3.45	0.0182	3.98
	B	0.462	0.0069	1.50	0.0180	3.90		

★(A) April to July, 1984 ($n=36$); the paracetamol sulphate eluted before paracetamol glucuronide; (B) July to September, 1984 ($n=65$); the paracetamol glucuronide eluted before paracetamol sulphate.
★★For 101 occasions combined.

65 occasions (July to September, 1984) a new column was used which reversed the order of elution of the sulphate and glucuronide without affecting the elution of the other metabolites. All subsequent columns resulted in the elution of the glucuronide before the sulphate. The estimates of the standard deviation and coefficient of variation for within-batch and total imprecision of paracetamol and five of its metabolites over the 101 occasions are given in Table I.

Dosing of subjects and storage of urine samples

Twelve male subjects, aged 18–48 years, each received a 1-g oral dose of paracetamol and urine samples were collected for 24 h. Subjects were allowed free access to food and drink (excluding alcohol) during the study. During the preliminary experiments it was observed that in some test urine samples which had been stored at room temperature for two to three days, there was a significant decrease in the concentration of 3-hydroxyparacetamol-3-sulphate, paracetamol-4-sulphate and paracetamol-4-glucuronide and an increase in free paracetamol on standing at room temperature. Since sulphatase and β-glucuronidase are known to be present in human urine [17], it was assumed that this was the cause of the problem but the presence of these enzymes was not confirmed. To overcome the problem saccharo-1,4-lactone (1 mg) and 0.5 *M* phosphate buffer (1 ml) were added to each urine sample bottle (50 ml capacity) used to store a sample of the

test urine after completion of collection. In addition sodium azide (1 mg) was added to inhibit any microbial growth. The addition of these compounds to each urine sample prevented metabolite hydrolysis even during several weeks of storage and did not interfere with the assay procedure. Test urine samples were routinely stored at −20 °C before analysis.

RESULTS AND DISCUSSION

Elution system

The six metabolites of paracetamol investigated in the present study possess a wide range of polarity. Accordingly, it was difficult to identify a reversed-phase chromatographic system which gave good peak symmetry and resolution whilst at the same time giving no interference from endogenous compounds in the urine. Paracetamol, its sulphate and cysteine conjugates and the catechol metabolite 3-hydroxyparacetamol-3-sulphate were the most difficult to resolve from endogenous compounds. Previously reported reversed-phase solvent systems for paracetamol and its metabolites have varied considerably in the identity of the organic solvent component, the pH and the presence of anionic or cationic ion-pairing agents. Our preliminary studies indicated that we were unlikely to develop a successful system based on previously published data mainly because of the unsatisfactory chromatography of the mercapturate or of the incomplete resolution of a metabolite and an endogenous compound. Cationic ion-pairing agents such as cetyltrimethylammonium and tetrabutylammonium were observed to delay the elution of paracetamol sulphate and glucuronide and accelerate the elution of the cysteine and mercapturate conjugates but tended to impair the resolution of the sulphate, glucuronide and cysteine conjugates. This observation was consistent with previous reports [9, 10]. The choice of pH of the eluent for ion-suppression chromatography was found to be crucial. The pH of previously published systems ranged from 2.1 [12] to 4.7 [16]. Above pH 3 we obtained incomplete resolution of paracetamol, its cysteine conjugate and endogenous compounds whilst at pH 2.5 the 3-hydroxyparacetamol-3-sulphate was incompletely resolved from paracetamol-4-glucuronide. Only at pH 2.0 were these resolution problems overcome. The chemical nature of the eluent was equally important. Thus both formic acid–acetonitrile–water and acetic acid–acetonitrile–water systems at pH 2.0 gave unacceptable peak resolution. However, the phosphate buffer–acetonitrile system completely resolved paracetamol, its six metabolites, the internal standard and endogenous compounds and gave good peak symmetry (Fig. 1). It was found that for the elution of all the compounds except the mercapturate conjugate the amount of acetonitrile in the eluent needed to be kept to a minimum. Since a dual-pump system was used which did not permit the introduction of the second pump after a period of isocratic elution, the proportion of solvent B in the eluent for the first 25 min was kept to the minimum consistent with reproducible performance. The subsequent linear gradient to 70% solvent B resulted in the elution of the mercapturate with good peak symmetry and with separation from endogenous compounds. The two major metabolites, paracetamol-4-glucuronide and paracetamol-4-sulphate, were adequately resolved from each other and from other

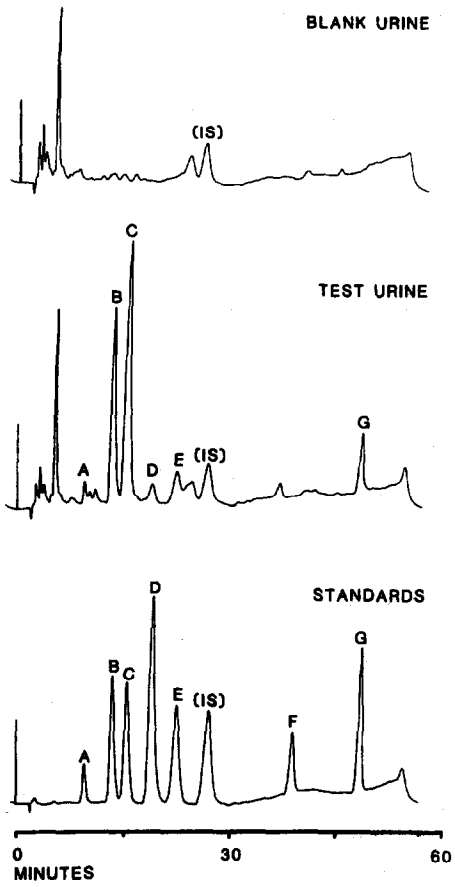

Fig. 1. Chromatograms of blank urine with added 3-hydroxyacetanilide, internal standard, test urine and standards of paracetamol and six metabolites. The chromatography was carried out on a Spherisorb ODS column with phosphate buffer (pH 2.0)-acetonitrile gradient elution. The test urine was a 24-h sample from a volunteer who had received a 1-g oral dose of the drug. Peaks: D = paracetamol; A = 3-hydroxyparacetamol-3-sulphate; B = paracetamol-4-glucuronide; C = paracetamol-4-sulphate; E = paracetamol-3-cysteine; IS = 3-hydroxyacetanilide, internal standard; F = 3-methoxyparacetamol-4-sulphate; G = paracetamol-3-mercapturate.

compounds to permit easy quantification of the minor excretory products.

The internal standard, 3-hydroxyacetanilide, eluted in 27.5 min. The relative retention time for paracetamol and its metabolites were: 3-hydroxyparacetamol-3-sulphate, 0.37; paracetamol-4-glucuronide, 0.53; paracetamol-4-sulphate, 0.62; paracetamol, 0.75; paracetamol-3-cysteine, 0.88; 3-methoxyparacetamol-4-sulphate, 1.45; paracetamol-3-mercapturate, 1.77. The total analysis time for each test sample was 52 min. This is a relatively long time and restricts the number of samples that can be analysed in a 24-h period to about 25. Even so, the advantages of the method over previously published ones commend it for future use. The order of elution of paracetamol-4-sulphate and paracetamol-4-glucuronide varied from column to column from the same manufacturer. At the beginning of the

study the sulphate conjugate eluted first, but on all subsequent Spherisorb ODS columns the order was reversed. The retention times for all the other compounds remained constant as did the quality of the resolution of all the peaks. As is clear from Table I the order of elution of the glucuronide and sulphate metabolites did not influence their quality control data or that of the other analytes.

Detection limits and precision

The minimum detectable on-column amount of paracetamol and its six metabolites in urine ranged from 10 ng for paracetamol (0.5 μg/ml for a 20-μl injection) to 50 ng for its mercapturate and cysteine conjugates (2.5 μg/ml). This is comparable with that reported by Wilson et al. [14] for UV detection but is five to ten times greater than that reported for the electrochemical detection of some of the metabolites [14, 16]. The within-batch and total imprecision data were outstanding with coefficients of variation for all analytes below 4%. No imprecision data are presented for the 3-methoxy-4-sulphate metabolite of paracetamol as it was not detectable in the test urine samples. Quality control data for this metabolite, obtained from blank urines to which samples of the synthetic metabolites had been added, were comparable with those for the other five metabolites. The life span of a column was in excess of 2000 h chromatography time. An important observation which emerged from the calibration curve data for paracetamol and its metabolites was that they did not have the same molar extinction coefficient at 254 nm, the values ranging from $5.3 \cdot 10^3$ l mol^{-1} cm^{-1} to $10.2 \cdot 10^3$ l mol^{-1} cm^{-1}. This confirms the need for individual calibration curves for each metabolite and emphasises that errors would arise if all the metabolites were assumed to behave as paracetamol. This observation is in contrast to that of Howie et al. [7]. Some previous workers have avoided the need to conduct individual calibration curves by utilising paracetamol metabolite response factors first calculated by Hart et al. [10].

Paracetamol metabolites after a therapeutic dose

The results of the application of the method to the 24-h urine samples from male volunteers who had received a 1-g therapeutic dose of the drug are presented in Table II. The data confirm previous work that the glucuronide and sulphate conjugates are responsible for the major part of paracetamol metabolism. Their relative proportions, however, were more variable than some previous reports have indicated [3, 15] and in some subjects the sulphate conjugate was quantitatively more important than the glucuronide. The mercapturate and cysteine conjugates were similar in quantitative importance in total generally accounting for 7–12% of the 1-g dose of paracetamol. Their ratio (0.4–1.7) was, however, somewhat smaller than previously reported [2, 3, 11]. 3-Hydroxyparacetamol-3-sulphate was found in all the test urine samples and accounted for up to 5% of the dose making it comparable in quantitative importance with each of the glutathione-derived conjugates. The presence of 3-hydroxyparacetamol-3-sulphate as a urinary metabolite was confirmed by its disappearance after treatment of the urine with sulphatase (*Helix pomatia*, Sigma, 37°C, 8 h) and also by its co-chromatography with added synthetic metabolite. The quantitative data for this

TABLE II

MEAN 24-h URINARY EXCRETION OF PARACETAMOL AND FIVE METABOLITES IN TWELVE HEALTHY MALE ADULTS EXPRESSED AS PERCENTAGE OF THE 1-g ORAL DOSE (6.67 mmol) OF PARACETAMOL

Analyte	Excretion (%)	
	Mean ± 1S.D.	Range
Paracetamol	3.3 ± 1.0	1.9- 5.4
Glucuronide	40.5 ±11.8	31.7- 64.7
Sulphate	37.2 ± 9.1	24.4- 57.0
Cysteine	4.3 ± 3.4	0.6- 13.7
Mercapturate	3.12± 1.8	0.5- 6.1
3-Hydroxysulphate	2.5 ± 8.6	0.4- 4.8
Recovery (%)	90.9± 8.6	82.3-102.2

metabolite indicate that it can no longer be regarded as a minor metabolite of little metabolic or toxicological importance particularly when its quantitative importance is coupled with its known hepatoxicity [18]. The method of Wilson et al. [14], which relied on the detection of the unconjugated form of this metabolite, suffered from the disadvantage that the free metabolite in their system co-eluted with some endogenous constituents of urine which were electrochemically active. This affected their detection limits for this metabolite. No similar problem was encountered in the present study. In view of the limitations to sulphate conjugation in man [19], it is interesting to note that the ratio of 3-hydroxyparacetamol-3-sulphate to paracetamol-4-sulphate in a given individual in the present study was consistently in the range of 0.07–0.13.

In none of the 24-h urine samples was 3-methoxyparacetamol-4-sulphate detected. In view of the detection limits for this metabolite, our results indicate that if it is present, as the results of Andrews et al. [2] indicate, it must account for less than 0.15% of a therapeutic dose. The study of Hamilton and Kissinger [12] on a 4-h urine sample following the oral administration of a 1-g dose indicated that this metabolite may be as quantitatively important as the cysteine conjugate. Their study also detected the glucuronide conjugate of this metabolite in amounts similar to that of the sulphate conjugate. This disparity between the results of the present study and those of Hamilton and Kissinger [12] for this methoxy metabolite warrant further studies, but it is interesting to note that previous non-quantitative studies of this metabolite [2, 8, 9, 14] have indicated that its importance may increase in overdose situations.

In some of the test urine samples, small unidentified peaks were observed. These were not present in the blank urine samples from the same subject and were shown not to be nicotine, caffeine or theobromine. The peaks were not sensitive to treatment of the urine with β-glucuronidase or sulphatase. They were not investigated further. No evidence was found for the presence of conjugates of 3-thiomethylparacetamol. This is consistent with the findings of Wilson et al. [14]

who could only detect the metabolite in the urine of a patient who had ingested an unknown but large quantity of the drug.

The five metabolites together with unchanged paracetamol accounted for 82.3–102.2% of the administered 1-g dose of paracetamol in 24 h. These values are very similar to the findings of Prescott et al. [11] and contrast with those of Miners et al. [15], who obtained essentially quantitative recovery with very much smaller inter-individual variation in the quantitative importance of individual metabolites even though they did not analyse for the catechol metabolites.

CONCLUSION

The present method allows the quantification of paracetamol and six of its metabolites in urine and is sensitive and precise. It has been applied to the analysis of paracetamol and its metabolites in several hundred urine samples. The work has been part of a study of the influence of sex, menstrual cycle and oral contraceptives on the rate and route of paracetamol metabolism in volunteers receiving a single oral therapeutic dose of the drug. The results of these studies will be presented in detail elsewhere. The method has also been used by independent workers to study the influence of the H_2-antagonist ranitidine on paracetamol metabolism [20].

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