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Note

Determination of phenacetin and its major metabolites in human plasma and urine by high-performance liquid chromatography

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Since phenacetin, a mild analgesic agent, has been withdrawn from pharmacies in many countries, the public interest has shifted to the use of acetaminophen. However, in some countries a large amount of phenacetin is still consumed and chronic interstitial nephritis was observed in many phenacetin abusers [1]. Phenacetin is metabolized mainly by dealkylation to acetaminophen in the hepatic microsomal enzyme system and also partly by deacetylation to *p*-phenetidine, a reaction catalysed by carboxyesterase [2]. Investigating the metabolism of phenacetin in man, with particular reference to the toxic effects on the kidney, only unchanged phenacetin and acetaminophen were estimated by thin-layer chromatography [3]. Recent analytical work has concentrated on the measurement of acetaminophen and its metabolites utilizing high-performance liquid chromatography (HPLC) for separation [4—6]. Other reported HPLC methods for the estimation of phenacetin and acetaminophen in plasma are complex, do not use an internal standard and give no details of reproducibility [7, 8]. In addition, the limit of detection was not very sufficient [9], extraction procedures were very complicated [10] and estimation of cysteine and mercapturic acid conjugates of acetaminophen was very problematic using electrochemical detection [10—12]. Furthermore, interfering peaks with endogenous substances in plasma and urine were not investigated. Based on these problems, in the present study an assay with the separation of five major metabolites of phenacetin and unchanged phenacetin in human plasma and urine has been developed utilizing HPLC.

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METHODS

High-performance liquid chromatography

HPLC was performed on a component system consisting of a Spectra-Physics Model SP 8100 liquid chromatograph solvent delivery system. The chromatograph was equipped with a commercially available reversed-phase column (25 cm × 4 mm I.D.) filled with LiChrosorb RP-18, 5 μm particle size (E. Merck, Darmstadt, F.R.G.). A fixed-wavelength UV detector (SP 8320) was operated at a wavelength of 254 nm. A computing integrator (SP 4100) was used to record the chromatograms and provide integration of the peak areas as well as calculation of the concentrations by the internal standard method. All injections were performed automatically using a Spectra-Physics SP 8110 autosampler together with a Valco-valve injector containing a 100-μl sample loop.

For measurement of the metabolites, a mobile phase of 1% glacial acetic acid–0.1 *M* potassium dihydrogen phosphate (3:97) was used, while for the unchanged phenacetin determination, methanol–1% acetic acid–0.1 *M* potassium dihydrogen phosphate (33:3:64) served as a mobile phase. The HPLC system was operated at a flow-rate of 1.2 ml/min and at a temperature of 45°C.

Phenacetin, acetaminophen, potassium dihydrogen phosphate, 4-fluorophenol (internal standard) and methanol were purchased from Merck. Glucuronide, sulphate, cysteine and mercapturic acid conjugates of acetaminophen were gifts from Sterling-Winthrop (U.K.).

Preparation of plasma and urine samples

Before and after administration of 900 mg of phenacetin blood samples (10 ml) were withdrawn from healthy volunteers through an indwelling cannula in the antebrachial vein at certain time intervals. Perchloric acid (5%; 0.5 ml) for deproteinization, which contained the internal standard (4-fluorophenol, 1 mg/ml), was added to 0.5 ml of each plasma sample. After mixing with a vortex mixer, the samples were centrifuged for 20 min at 2800 *g*. The supernatant was filtered through a 0.2-μm disposable filter (Acrodisc) and 100 μl were injected onto the column.

TABLE I

CONCENTRATION RANGE, REGRESSION EQUATIONS AND CORRELATION COEFFICIENTS OF UNCHANGED PHENACETIN AND ITS MAJOR METABOLITES

y = peak area of compound; x = μg of compound.

Compound	Range (μg/ml)	Regression equation	Correlation coefficient
Phenacetin	0.01— 1.3	$y = (138x + 1.5) \cdot 10^3$	0.99
Acetaminophen	0.02— 2.5	$y = (120x - 0.3) \cdot 10^3$	1.0
Acetaminophen glucuronide	0.02—164	$y = (16x + 33.3) \cdot 10^3$	0.99
Acetaminophen sulphate	0.04—140	$y = (14x - 1.1) \cdot 10^3$	0.99
Acetaminophen cysteine	0.04— 2.5	$y = (640x - 0.5) \cdot 10^3$	0.99
Acetaminophen mercapturic acid	0.05— 2.5	$y = (48x - 2.0) \cdot 10^3$	0.99

Urine samples collected from healthy volunteers before and after phenacetin administration were used, and 4-fluorophenol (1 mg/ml) as an internal standard was added to each urine sample. All samples were diluted 20 times with distilled water and then 100-μl aliquots were injected directly onto the column.

Linearity studies

The linearity of the detector was evaluated by measuring six different standard concentrations of phenacetin and its metabolites in plasma and urine samples (Table I). Standard curves were fitted by computer-assisted regression analysis using the method of least-squares.

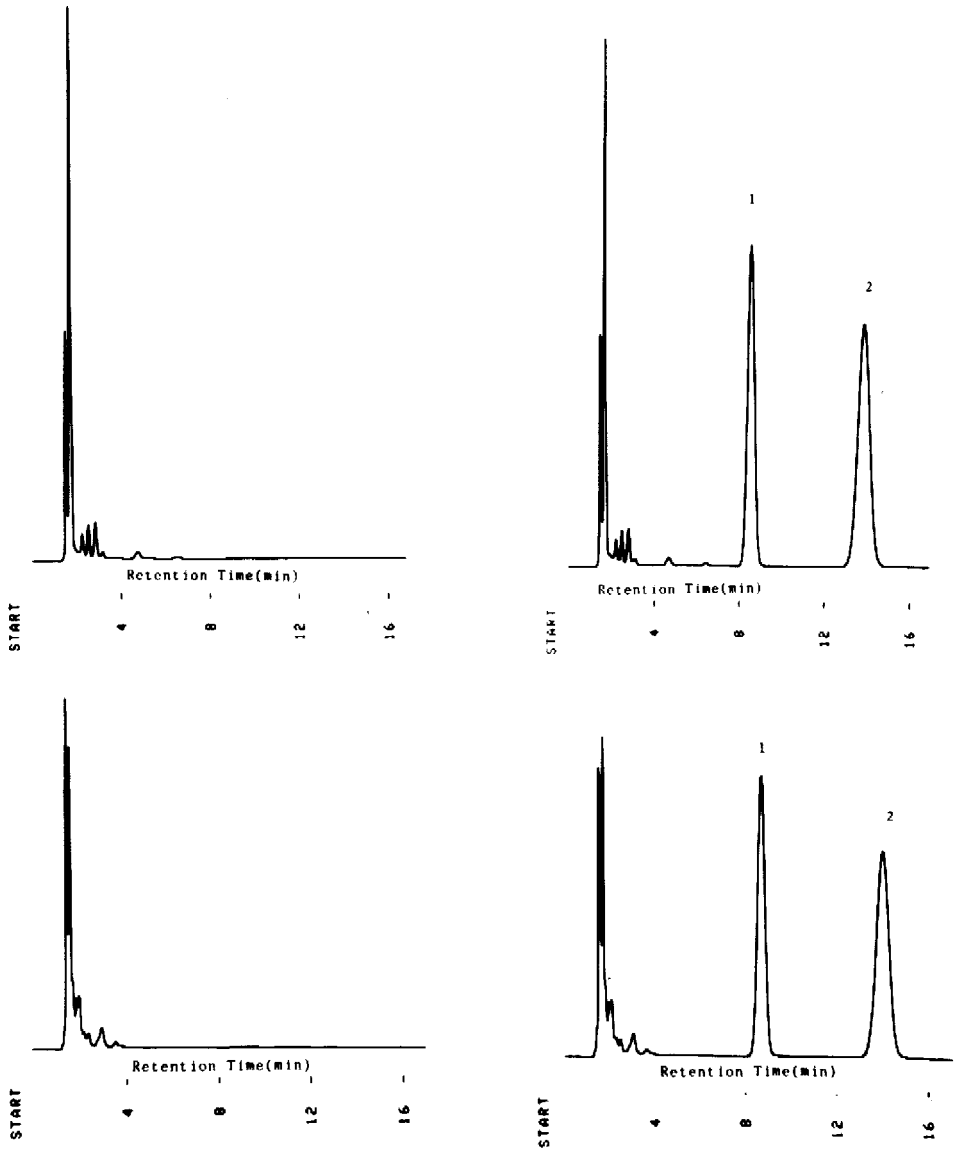

Fig. 1. Chromatographic separation of phenacetin in human blank plasma (upper panel) and urine (lower panel). 1 = Internal standard (8.74 μg/ml); 2 = phenacetin (3.75 μg/ml).

TABLE II

RECOVERY, REPRODUCIBILITY AND ACCURACY OF UNCHANGED PHENACETIN AND ITS MAJOR METABOLITES IN PLASMA AND URINE

Phe = Phenacetin; A = acetaminophen; G = acetaminophen glucuronide; S = acetaminophen sulphate; C = acetaminophen cysteine; M = acetaminophen mercapturic acid.

			Mean ± S.D.					
			Phe	A	G	S	C	M
Plasma (n = 5)	Recovery (%)	Within-day	99.78 ± 0.06	98.45 ± 0.87	98.19 ± 1.29	99.39 ± 0.76	98.19 ± 1.44	98.67 ± 1.22
		Day-to-day	99.79 ± 0.12	98.70 ± 0.83	99.53 ± 0.42	98.66 ± 1.77	99.53 ± 0.36	98.76 ± 1.01
	Reproducibility	Within-day	0.06	0.88	1.31	0.76	1.46	1.23
		Day-to-day	0.12	0.84	0.42	1.79	0.36	1.01
	Accuracy (%)	Within-day	1.36 ± 0.49	0.47 ± 0.15	1.54 ± 0.59	0.40 ± 0.13	0.57 ± 0.22	0.70 ± 0.18
		Day-to-day	1.57 ± 0.67	1.52 ± 0.19	1.26 ± 0.67	0.43 ± 0.03	0.60 ± 0.21	1.97 ± 1.41
Urine (n = 5)	Recovery (%)	Within-day	99.41 ± 0.48	97.56 ± 0.19	99.15 ± 1.11	99.79 ± 0.13	97.66 ± 0.96	97.7 ± 0.33
		Day-to-day	98.54 ± 1.80	97.36 ± 1.94	97.51 ± 1.38	99.64 ± 0.07	96.11 ± 2.04	97.26 ± 1.87
	Reproducibility	Within-day	0.48	0.20	1.20	0.13	0.98	0.34
		Day-to-day	1.83	1.99	1.42	0.07	2.12	1.92
	Accuracy (%)	Within-day	0.73 ± 0.24	0.50 ± 0.13	0.99 ± 0.34	0.58 ± 0.15	0.51 ± 0.16	0.61 ± 0.15
		Day-to-day	1.10 ± 0.57	1.37 ± 0.72	0.85 ± 0.10	1.13 ± 0.79	0.70 ± 0.15	1.13 ± 0.36

Recovery, reproducibility, accuracy

Recovery studies were carried out in plasma and urine obtained from volunteers by adding standard solutions of phenacetin and its metabolites in the same concentrations as used in the linearity studies (Table I), whereby within-day and day-to-day measurements were performed.

Recovery was calculated (in %) from the concentrations measured in plasma and urine divided by the concentrations of the standard solutions. Reproducibility was determined as a relative standard deviation (expressed in %), dividing the standard deviation by the mean of all individual measurements. The difference between the actual amount of substances added to plasma or urine and the amount measured, divided by the actual amount, was used for the calculation of accuracy (in %).

RESULTS

In Figs. 1 and 2, the typical chromatograms before and after phenacetin

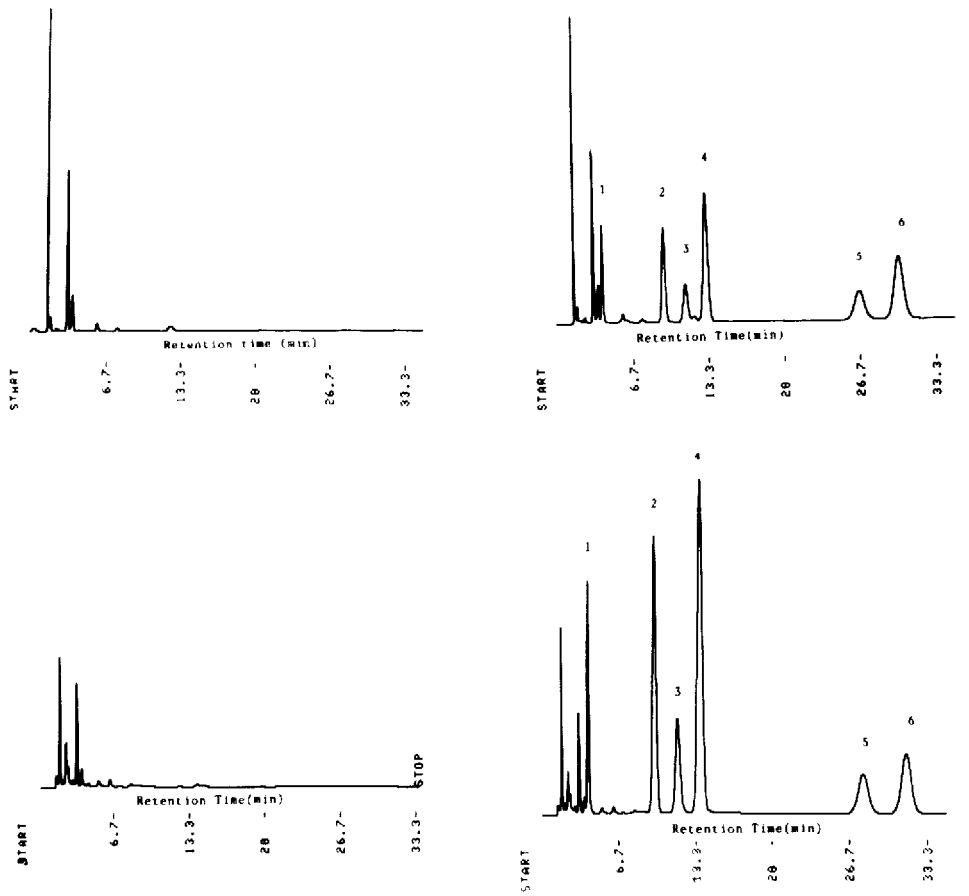

Fig. 2. Chromatographic separation of phenacetin metabolites in human blank plasma and urine following phenacetin administration. 1 = Acetaminophen glucuronide (0.17 μg/ml); 2 = acetaminophen sulphate (0.46 μg/ml); 3 = acetaminophen cysteine (0.25 μg/ml); 4 = acetaminophen (0.41 μg/ml); 5 = acetaminophen mercapturic acid (0.21 μg/ml); 6 = internal standard (4.37 μg/ml).

administration to healthy volunteers are shown. These chromatograms are identical to those obtained from standard solutions of phenacetin and its metabolites in blank plasma or urine. The separation and elution times of phenacetin, its metabolites and the internal standard in plasma and urine samples are also seen in these figures. The assay for phenacetin can be completed within 10 min, and in 35 min for the five metabolites investigated.

The regression equations for each individual compound in the different concentrations used, and the correlation coefficients calculated, are shown in Table I. The recovery, reproducibility and accuracy of unchanged phenacetin and its major metabolites are presented in Table II. The practical limit of sensitivity for phenacetin and the five major metabolites was 20—50 ng/ml.

DISCUSSION

Analgesic abuse, in particular with phenacetin, may result in chronic renal failure [1] and therefore a sensitive method for the determination of phenacetin and its metabolites is required to elucidate the mechanism of renal toxicity. In addition, a sensitive and rapid method for the estimation of phenacetin in plasma and urine should be available for the detection of abusers in certain patient populations. In the present study, an isocratic HPLC procedure for the separation of phenacetin alone or its five major metabolites in plasma and urine was established, in which no time-consuming extraction procedure is necessary, a simple mobile phase is employed and a good reproducibility with minimum detectable limits at 20- 50 ng/ml is achieved. Using 4-fluorophenol as internal standard, a sufficient extraction and complete separation from phenacetin and its metabolites is obtained, as found previously [8], which supports its use as an internal standard. In addition, no endogenous substances in plasma and urine of healthy volunteers were found to interfere with this method. Utilizing this HPLC method in patient studies of renal failure, no interference with other drugs or substances usually administered or eventually accumulating in this disease was observed [13]. In addition, no interaction was seen after co-administration of phenacetin and salicylates, two drugs often taken together in analgesic drug abuse [14]. However, on screening multiple-drug abusers for phenacetin, an interference may occur despite the fact that none has been observed so far in our laboratory. Therefore, under certain experimental conditions using other drugs that have not been tested, interference should be excluded using this analytical method.

In conclusion, based on the data presented in this study, the method developed can be utilized to elucidate further the metabolism of phenacetin and its connection to the mechanism of renal toxicity.

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