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# Analysis of phenprocoumon and its hydroxylated and conjugated metabolites in human urine by high-performance liquid chromatography after solid-phase extraction

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## ABSTRACT

The anticoagulant phenprocoumon is mainly metabolized in humans to hydroxylated metabolites and their glucuronides. A method is described for the determination of phenprocoumon, 4'-hydroxyphenprocoumon, 6-hydroxyphenprocoumon, 7-hydroxyphenprocoumon, and their glucuronide and sulphate conjugates in human urine. Reversed-phase high-performance liquid chromatography is performed after selective extraction with disposable quaternary amine columns of untreated, and β-glucuronidase- or sulphatase-treated urine samples. Urinary excretion data are presented for total, glucuronidated, sulphated and free phenprocoumon, 4'-hydroxyphenprocoumon, 6-hydroxyphenprocoumon and 7-hydroxyphenprocoumon in twelve patients after an average daily dosage of 1.3–4.2 mg phenprocoumon.

## INTRODUCTION

The oral anticoagulant phenprocoumon (PPC, Fig. 1), 4-hydroxy-3-(1-phenylpropyl)-2*H*-1-benzopyran-2-one, is metabolized in humans to hydroxylated me-

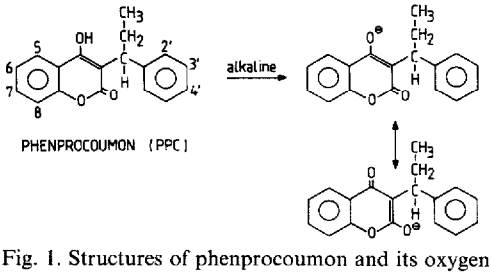


Fig. 1. Structures of phenprocoumon and its oxygen anion tautomers.

tabolites and their conjugates. The major unconjugated metabolite in urine is 7-hydroxyphenprocoumon (7-OH-PPC). Minor metabolites are 4′- and 6-hydroxyphenprocoumon (4′-OH-PPC, 6-OH-PPC). Studies with gas chromatography–mass spectrometry (GC–MS) indicate that both PPC and the hydroxy metabolites are extensively conjugated prior to excretion into the urine. The identity of the conjugates could not be unambiguously assessed because hydrolysis was conducted with a crude enzyme preparation containing both glucuronidase and sulphatase activities [1,2].

In the quantification of conjugates, enzymic hydrolysis by glucuronidase or sulphatase may give rise to non-reproducible rates of hydrolysis because urine samples may contain unknown concentrations of inhibitors of these enzymes. In urine, glucarolactone, for example, inhibits glucuronidase whereas phosphate and sulphate ions are strong inhibitors of sulphatases A and B [3].

To define the basic pharmacokinetic and metabolic parameters of PPC in patients on chronic anticoagulant therapy for interaction studies between PPC and enzyme-inducing drugs, it was necessary to develop an assay for PPC, its hydroxy metabolites and their conjugates in urine. Methods using fluorimetry, thin-layer chromatography, gas chromatography and high-performance liquid chromatography (HPLC) have been described for the analysis of PPC in serum, plasma or urine [4,5]. However, efforts to analyse free and conjugated PPC and its hydroxy metabolites in urine with HPLC were unsuccessful, owing to interference from endogenous compounds and poor analytical sensitivity [1,2]. One published method for analysis of PPC and metabolites in urine with GC–MS operating in the selected-ion monitoring mode is semi-quantitative [1]; another [2] lacks analytical information such as reproducibility and sensitivity.

This paper describes a method for the determination of PPC, 4′-OH-PPC, 6-OH-PPC, 7-OH-PPC, and their glucuronide and sulphate conjugates in human urine, involving reversed-phase HPLC after selective extraction with disposable quaternary amine columns of untreated and β-glucuronidase- or sulphatase-treated urine samples.

No standards of the glucuronide and sulphate conjugates were available for finding optimal conditions for hydrolysis of the conjugates of PPC and its hydroxy metabolites. For these studies, therefore, we used incubation experiments with conjugated metabolites of another coumarin analogue, 4-methylumbelliferone, and fluorescence measurement. We studied the percentage hydrolysis of 4-methylumbelliferyl sulphate and 4-methylumbelliferyl-β-glucuronide toward a specific sulphatase (Type VI) and a specific glucuronidase (Type B-1) in distilled water and urine. In addition we studied the intrinsic hydrolytic activity of endogenous enzymes in urine towards the 4-methylumbelliferyl conjugates.

## EXPERIMENTAL

### *Standards and reagents*

All chemicals were of analytical grade. Citric acid, diammonium hydrogencitrate, 0.067 *M* phosphate buffer (Titrisol pH 7.0), glacial acetic acid, sodium acetate trihydrate, sodium hydroxide, ammonium hydroxide, methanol and hexane (Uvasol) were purchased from Merck (Darmstadt, F.R.G.), acetonitrile (Chromar grade) from Mallinckrodt (Paris, KY, U.S.A.) and tetrahydrofuran (HPLC grade, unstabilized) from Fisons (Loughborough, U.K.).

Sulphatases Type VI (from *Aerobacter aerogenes*), Type H-1 (from *Helix pomatia*) and Type V (from *Patella vulgata*) and β-glucuronidases Type B-1, Type B-10 and Glucurase® (all from bovine liver) were obtained from Sigma (St. Louis, MO, U.S.A).

4-Methylumbelliferyl sulphate, 4-methylumbelliferyl-β-glucuronide and 4-methylumbelliferone were from Koch-Light Labs. (Colnbrook, U.K.). Stock solutions of 4-methylumbelliferone and 4-methylumbelliferyl sulphate in methanol and of 4-methylumbelliferyl-β-glucuronide in methanol–water–acetone (5:5:1, v/v) were prepared to give a concentration of 20 m*M*. Disposable quaternary amine 3-ml extraction columns were purchased from Baker (Phillipsburg, NJ, U.S.A.). PPC was a gift from Hoffmann-La Roche (Mijdrecht, The Netherlands). The pure hydroxy metabolites of PPC, 4′-OH-PPC, 5-OH-PPC, 6-OH-PPC, 7-OH-PPC and 8-OH-PPC were synthesized by Professor W. F. Trager (Department of Medicinal Chemistry, School of Pharmacy, University of Washington, Seattle, WA, U.S.A.). Stock solutions of PPC and its hydroxy metabolites were prepared in ethanol to give a concentration of 1 g/l. For analyses of unconjugated and unconjugated plus conjugated PPC and metabolites, urine standards of 25, 50, 75, 100, 180, 250 and 500 μg/l, respectively, were prepared by spiking PPC, 4′-OH-PPC, 6-OH-PPC and 7-OH-PPC to pooled fresh urine samples from volunteers who did not use any medication, and stored at −20°C.

An acetate buffer (pH 5.0) was prepared by dissolving 150 g of sodium acetate trihydrate in 500 ml of distilled water and adjusting the pH to 5.0 with glacial acetic acid. A 0.2 *M* carbonate–glycine buffer (pH 10.5) was prepared by diluting 200 ml of 0.5 *M* $Na_2CO_3$ with 200 ml of distilled water, adding 0.5 *M* glycine till pH 10.5 and adjusting the volume to 500 ml.

### *Equipment*

The vacuum manifold was a PVC box with twelve-place moulded cover with luer fittings and gasket, manufactured in our laboratory. The chromatograph consisted of a microprocessor-controlled HPLC solvent-delivery system (Model SP 8700, Spectra Physics, San Jose, CA, U.S.A.), UV variable-wavelength detector (GM 770, Schoeffel, Westwood, NJ, U.S.A.) and a syringe-loading sample injector with a 175-μl sample loop (Model 7125, Rheodyne, Cotati, CA, U.S.A.) The chromatogram was registered by a flat-bed recorder (Model BD 41, Kipp,

Delft, The Netherlands), with a measuring range of 10–50 mV and a chart speed of 0.5 cm/min. The column was a 12.5 cm × 4.6 mm I.D. Hyperchrome column (Bischoff, Stuttgart, F.R.G.), packed upwardly in our laboratory with ODS-Hypersil, 3 μm particle size (Shandon, Astmoor, Runcorn, U.K.) by a stirred-slurry column packer (Model 705, Micromeritics, Norcross, GA, U.S.A.) [6]. The column was eluted isocratically at ambient temperature (range 20–22°C) with a solvent system of acetonitrile–methanol–tetrahydrofuran–10% (v/v) acetic acid (pH 2.7) (27:14:2:57, v/v) at 1.2 ml/min. The eluate absorbance was measured at 312 nm, at a detector sensitivity of 0.01 A full-scale.

In studies on the hydrolysis of 4-methylumbelliferone conjugates, fluorescence of the liberated 4-methylumbelliferone was measured in a fluorimeter (Model 204A, Perkin Elmer, Norwalk, CT, U.S.A.) at excitation and emission wavelengths of 365 and 450 nm, respectively.

*Patients*

Six male and six female outpatients in the age range between 43 and 69 years, who gave consent to the study, received orally a mean dosage of 1.3–4.2 mg PPC. The PPC dosage was stable for at least six weeks before the study. Only benzodiazepines were accepted as concomitant medications. The patients had a normal liver and renal function. Urine was collected for 24 h, and a sample was frozen at −20°C till analysis.

*Hydrolysis of conjugates of 4-methylumbelliferone*

In one series of experiments, mixtures with a final volume of 0.30 ml containing enzyme and various substrate concentrations of a methylumbelliferyl conjugate in 0.33 *M* acetate buffer (pH 5.0) were incubated for 60 min at 37°C in both distilled water and drug-free urine. The incubates contained *ca.* 0.2 U of sulphatase or 40 U of glucuronidase. The manufacturer defines 1 U of sulphatase as hydrolysing 1.0 μmol of nitrophenylsulphate per minute at pH 7.1 and 37°C and 1 U of glucuronidase as liberating 1.0 μg of phenolphthalein per hour from its glucuronide at pH 5.0 and 37°C.

In another series of experiments, standards containing 0, 0.4, 0.8, 1.2 and 1.6 m*M* 4-methylumbelliferyl sulphate or 4-methylumbelliferyl-β-glucuronide in drug-free urine were incubated as described in *Pre-treatment of urine samples* for glucuronide and sulphate conjugates. After incubation, the glucuronidase- and the sulphatase-treated incubates were diluted 1:100 and 1:300 (v/v) with distilled water, respectively. In both series of experiments 2.7 ml of 0.2 *M* carbonate–glycine buffer (pH 10.5) were added to 0.3 ml of the (diluted) incubates, and the fluorescence of the liberated 4-methylumbelliferone was measured. Mixtures of urine, enzymes and substrate in the same amounts as in the incubates were used as standards, but the carbonate–glycine buffer was added before the substrate. To these mixtures various amounts of 4-methylumbelliferone were added. Mixtures without added substrate served as a blank.

*Pre-cleaning of the extraction columns*

During pre-cleaning and extraction the columns were placed onto the vacuum manifold, and eluent flow was controlled at 2–3 ml/min by means of a valve. The columns were conditioned by aspiration of 3 ml of hexane followed by air-drying under vacuum for 1–3 min. This step was followed by sucking 3 ml of methanol, 3 ml of distilled water and 3 ml of 0.067 *M* phosphate buffer (pH 7.0), respectively, without air-drying.

*Pre-treatment of urine samples*

For determination of the glucuronide conjugates, 1 ml of urine samples from patients and standard urine samples were diluted with 1 ml of distilled water. After adjustment to pH 5.0 with acetate buffer (pH 5.0), the solution was incubated for 16 h at 37°C with 50 μl of β-glucuronidase (Type B-1; 8250 U/ml) solution. For analysis of the sulphate conjugates, 2 ml of urine were incubated for 16 h at 37°C and with 100 μl of sulphatase (Type VI; 16.7 U/ml) solution, after adjustment to pH 6.5–7 with 0.3 *M* ammonia. For determination of unconjugated PPC and metabolites 2 ml of urine were used.

*Extraction of urine samples*

Before column extraction all (incubated) urine samples were adjusted to pH 6.5–7 with 0.3 *M* ammonia. The portions of pretreated urines were quantitatively added to the top of pre-cleaned quaternary amine columns, and a vacuum was applied. Then the columns were washed with 3 ml of 0.3 *M* ammonium formate (pH 8.0) followed by 3 ml of distilled water.

For determination of unconjugated PPC and metabolites only one further washing step, with 1 ml of 0.1 *M* diammonium hydrogencitrate (pH 3.5)–methanol (7:3; v/v), was necessary. For analyses of the conjugated compounds, two extra cleaning steps were performed with 1 ml of tetrahydrofuran (THF) and 1.5 ml of 0.1 *M* diammonium hydrogencitrate (pH 3.5)–methanol (7:3, v/v). After these cleaning steps the PPC compounds were eluted from the column into clean tubes with 2 ml of THF. The THF solution was evaporated to dryness under nitrogen at 50°C. The residue was reconstituted in 200 μl of mobile phase and 75 μl were injected for analysis of PPC, 4′-OH-PPC and 7-OH-PPC. For the determination of 6-OH-PPC, evaporation for 1.5 h at 60°C was necessary.

*Calibration curves*

Quantification of PPC and metabolites in the urine samples was made by reference to a standard calibration curve constructed with each batch of samples analysed. The standard curve was obtained from analysis of 1 or 2 ml of drug-free urine spiked with PPC, 4′-OH-PPC, 6-OH-PPC and 7-OH-PPC. The peak heights of these compounds were plotted against the concentration in urine.

## RESULTS AND DISCUSSION

### *Incubation experiments*

Incubation experiments with 4-methylumbelliferyl conjugates and three β-glucuronidases (Type B-1, B-10, Glucurase) and three sulphatases showed that, of the compounds under study, Type B-1 glucuronidase and Type VI sulphatase were the most specific (unpublished data). These enzymes were used further. After incubation of known amounts (range 0–15 $\mu M$) of 4-methylumbelliferyl conjugates in urine or distilled water with Type B-1 glucuronidase or Type VI sulphatase, the amount of liberated 4-methylumbelliferone was compared with that expected for 100% hydrolysis. The results are presented in Fig. 2. With the proper blanks the assay of glucuronides in urine was without problems. For the sulphates the situation was more complex. In aqueous medium we could not obtain 100% hydrolysis. Compared with its activity in an aqueous medium, the enzyme in urine was inhibited by *ca.* 30% as shown in the figure. In the presence of urine the rate of hydrolysis was linear for at least 60 min, and addition of 4-methylumbelliferyl sulphate indicated a linearity with substrate to over 15 $\mu M$. From these findings it was expected that hydrolysis with 0.4 kU of glucuronidase and 0.84 U of sulphatase per ml of urine for 16 h at 37°C should be appropriate for complete conversion of the phenprocoumon conjugates in concentrations up to 500 μg/l. To check this supposition, standards of 4-methylumbelliferyl conjugates (range 0–1.6 m$M$) in drug-free urine were incubated under these hydrolytic conditions. From the amount of liberated 4-methylumbelliferone it was deter-

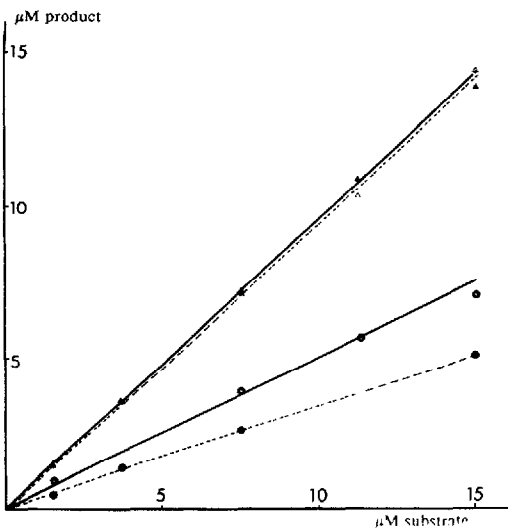


Fig. 2. Relation between the concentration ($\mu M$) of liberated 4-methylumbelliferone and the concentration ($\mu M$) of its conjugates added as substrate after incubation in the presence of (△,▲) glucuronidase or (○,●) sulphatase and in the absence (△,○) or presence (▲,●) of urine.

mined that the 4-methylumbelliferyl conjugates were hydrolysed for more than 96% with a linear rate to over 1.6 m*M* of substrate. A higher temperature of incubation was not used because chemical hydrolysis of the glucuronide conjugates made it impossible to quantify the sulphate conjugates.

Hydrolytic activity of endogenous urinary enzymes towards the conjugates was determined by measuring the formation of free products after incubation of urine with the conjugated substrates. Incubation showed no urinary glucuronidase activity towards the 4-methylumbelliferyl glucuronide. The sulphate, however, was hydrolysed to an appreciable extent, *ca.* 6%, when 4-methylumbelliferyl sulphate in a final concentration range of 0.0–0.1 m*M* was added to urine and incubated without enzyme. This finding suggests that sulphate conjugates of coumarins are possibly hydrolysed in the bladder, and that quantitative assay of sulphates in urine may not be a correct measure for the renal excretion of its sulphated metabolites.

*Extraction procedure*

Under alkaline conditions selective retention of PPC and metabolites on silica modified by bonding with a quaternary amine group proved to be successful, probably owing to ion-pair formation with oxygen anions (Fig. 1). To eliminate analytical variations between different batches of the disposable columns all extractions were performed with columns from the same batch (Lot No. 22762).

Several cleaning steps were necessary for optimal removal of interfering endogenous urine compounds. Under acidic conditions at the cleaning step with citrate buffer (pH 3.5)–methanol, PPC and metabolites already migrate in the column. Therefore its volume was very critical. This step proved to be necessary for elimination of an interfering endogenous component from urine and for attaining high recovery in the THF fraction, as shown in Table I. Owing to the high boiling point of THF it took 30 min to evaporate this solvent. When the tube was dry it had to be removed from the heating-block immediately for a reliable analysis of PPC, 7-OH-PPC and 4′-OH-PPC. An endogenous urine compound with the same capacity factor interfered with 6-OH-PPC, and it was not possible to quantify 6-OH-PPC reliably under these conditions. However, this interfering component could be converted into another product after evaporation of the THF extract for 1.5 h at 60°C. Unfortunately this thermally converted compound had the same capacity factor as 4′-OH-PPC. This means that two analytical procedures were necessary to quantify PPC, 4′-OH-PPC, 6-OH-PPC and 7-OH-PPC. Wheeler *et al.* [7] described a solvent-dependent decomposition of 7-OH-PPC in the presence of oxygen. In our assay such instability of 7-OH-PPC was not observed.

Warfarin and acenocoumarol were tested as possible internal standards, but their recovery proved to be low and therefore unreproducible, and the method was further developed without an internal standard.

In order to determine the precision and the accuracy of the entire procedure

TABLE I

ANALYTICAL RECOVERY, ACCURACY, PRECISION AND DETECTION LIMIT FOR PPC AND METABOLITES

| | Method[a] | Recovery[b] (%) | Intra-assay precision | | | Inter-assay precision | | | Detection limit[c] (μg/l) |
|---|---|---|---|---|---|---|---|---|---|
| | | | Mean (μg/l) | C.V. (%) | *n* | Mean (μg/l) | C.V. (%) | *n* | |
| PPC | A | 90.3 | 40.3 | 5.7 | 8 | 44.2 | 6.9 | 4 | 8 |
| 4'-OH-PPC | A | 81.8 | 39.2 | 6.9 | 8 | 42.8 | 1.7 | 4 | 4 |
| 6-OH-PPC | A | 127.0 | 58.5 | 15.0 | 8 | –[d] | –[d] | –[d] | –[d] |
| 7-OH-PPC | A | 91.0 | 41.3 | 1.3 | 8 | 43.2 | 3.5 | 4 | 3 |
| PPC | B | 63.3 | 100.6 | 11.5 | 8 | 94.4 | 4.7 | 5 | 15 |
| 4'-OH-PPC | B | 72.6 | 104.9 | 4.2 | 8 | 98.3 | 2.3 | 5 | 9 |
| 6-OH-PPC | B | 79.8 | 93.4 | 10.9 | 8 | –[d] | –[d] | –[d] | 38 |
| 7-OH-PPC | B | 69.3 | 100.8 | 6.5 | 8 | 105.7 | 11.0 | 5 | 6 |
| PPC | C | 75.7 | 42.7 | 7.1 | 8 | 41.8 | 5.4 | 4 | 9 |
| 4'-OH-PPC | C | 76.7 | 39.0 | 6.9 | 8 | 41.6 | 3.7 | 4 | 4 |
| 6-OH-PPC | C | 104.0 | 42.0 | 16.4 | 8 | –[d] | –[d] | –[d] | 13 |
| 7-OH-PPC | C | 83.6 | 40.0 | 5.8 | 8 | 42.2 | 5.4 | 4 | 4 |

[a] Pretreatment and extraction procedure applied: (A) for unconjugated compounds; (B) for glucuronidated compounds; (C) for sulphated compounds.

[b] Recovery measured in triplicate as the ratio of the peak height of the compound in extracted urine standards (methods A and C: 40 μg/l; method B: 100 μg/l), to the peak height of a 100% solution in the mobile phase.

[c] At a signal-to-noise ratio of 3:1.

[d] Not determined.

for analysis of unconjugated and sulphated PPC, 4'-OH-PCC, 6-OH-PPC and 7-OH-PPC, urine samples containing 40 μg/l unconjugated compounds were analysed on the same day and on different days. For the procedure with glucuronidase, urine samples containing 100 μg/l unconjugated compounds were used. The results are shown in Table I. The detection limits, also shown in this table, were defined as the concentration of the compound necessary to produce a signal three times stronger than the background noise under assay conditions. The relatively high coefficient of variation and detection limit for 6-OH-PPC after pre-treatment with β-glucuronidase and sulphatase in comparison with PPC, 4'-OH-PPC and 7-OH-PPC must be associated with a remaining minor amount of the interfering compound, which was not thermally converted. Free 6-OH-PPC could not be detected in urine from patients receiving a daily dosage between 1.3 and 4.2 mg PPC. A linear correlation between peak height and the concentrations of PPC, 4'-OH-PPC, 6-OH-PPC and 7-OH-PPC was found in the range 25–250 μg/l with the analytical procedure for the unconjugated and sulphated compounds, and in the range 50–500 μg/l with the procedure for the glucuronide conjugates.

*Chromatographic conditions*

As shown from the capacity factors of PPC and its hydroxy metabolites in Table II, separation of all compounds was obtained with the eluent methanol–10% (v/v) acetic acid (pH 2.2) (55:45, v/v). Under these chromatographic conditions no 5-OH-PPC or 8-OH-PPC could be detected in the β-glucuronidase-treated urine of twelve patients receiving chronic PPC with a mean daily dosage between 1.3 and 4.2 mg. However, under these conditions an endogenous urine compound interfered with 4'-OH-PPC. In the definitive procedure the mobile phase of acetonitrile–methanol–tetrahydrofuran–10% (v/v) acetic acid (pH 2.7) (27:14:2:57, v/v) was chosen for optimum resolution between PPC and its metabolites and endogenous urine compounds.

TABLE II

CAPACITY FACTORS OF PPC AND METABOLITES, AND SPECTRAL DATA IN THE MOBILE PHASE

| Compound | $k'^a$ | $k'^b$ | $\lambda_{max}$ (nm)[a] | log $\varepsilon_{312}$ |
|---|---|---|---|---|
| 4'-OH-PPC | 2.77 | 3.47 | 275, 282, 309, 319 (sh) | 4.13 |
| 5-OH-PPC | 4.03 | 13.03 | 303 | 4.06 |
| 6-OH-PPC | 3.67 | 4.76 | 280, 290 (sh), 329 | 3.69 |
| 7-OH-PPC | 4.23 | 5.57 | 276 (sh), 286, 317, 328 (sh) | 4.25 |
| 8-OH-PPC | 4.23 | 5.98 | 291 | 3.98 |
| PPC | 7.85 | 8.86 | 275 (sh), 285, 302 (sh), 309, 319 (sh) | 4.10 |

[a] Capacity factors and spectral data with acetonitrile–methanol–tetrahydrofuran–10% (v/v) acetic acid (pH 2.7) (27:14:2:57, v/v); sh = shoulder.

[b] Capacity factors with methanol–10% (v/v) acetic acid (pH 2.2) (55:45, v/v).

TABLE III

DRUGS INVESTIGATED FOR POSSIBLE INTERFERENCE WITH THE ASSAY OF PPC AND METABOLITES

| Name | $k'$[a] |
|---|---|
| Acenocoumarin | 5.07 |
| Acetaminophen | –[b] |
| Acetophenetidin | –[b] |
| Acetylsalicylic acid | 0.64 |
| Carbamazepine | 1.73 |
| Chlordiazepoxide | 5.56 |
| Desmethyldiazepam | 3.93 |
| Demoxepam | 1.59 |
| Diazepam | 5.63 |
| Diphenylhydantoin | 1.61 |
| Ethylbiscoumacetate | 1.09 |
| Flunitrazepam | 2.46 |
| Glaphenine | 1.24 |
| Lorazepam | 2.66 |
| Methaqualone | 2.33 |
| 8-Methoxypsoralen | 1.63 |
| Nitrazepam | 1.99 |
| Oxazepam | 2.49 |
| Phenobarbital | –[b] |
| Salicylic acid | 0.64 |
| Temazepam | 3.57 |
| Warfarin | 5.53 |

[a] Capacity factors with acetonitrile–methanol–tetrahydrofuran–10% (v/v) acetic acid (pH 2.7) (27:14:2:57, v/v).

[b] Not visible in chromatogram.

UV spectra between 250 and 350 nm were obtained under these conditions, and the wavelengths of maximum absorbance are listed in Table II. These are comparable with data for an acidic ethanolic solution [8]. For optimum sensitivity in the analysis of the relevant compounds a wavelength of 312 nm was chosen.

Table III lists 22 drugs, which were investigated for possible interference with PPC and its hydroxy metabolites in the selected chromatographic system. Desmethyldiazepam and temazepam may interfere with 5-OH-PPC and 6-OH-PPC, respectively. However, these drugs could not be isolated with the developed extraction procedure.

*Application*

Urine samples from twelve patients receiving PPC with a mean daily dosage of 1.3–4.2 mg were analysed. Fig. 3 shows chromatograms of a test mixture, an extract of a standard urine containing 100 μg/l 4'-OH-PPC, 6-OH-PPC, 7-OH-

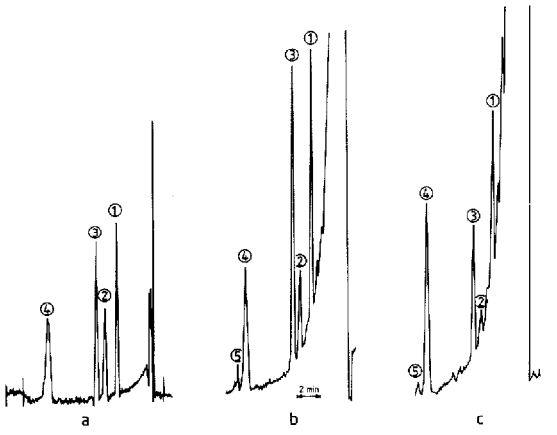


Fig. 3. Chromatograms of PPC and metabolites with the eluent acetonitrile–methanol–tetrahydrofuran–10% (v/v) acetic acid (pH 2.7) (27:14:2:57, v/v). (a) A test mixture of 4′-OH-PPC, 6-OH-PPC, 7-OH-PPC and PPC (40 ng each) at a detector sensitivity of 0.02 a.u.f.s.; (b) a standard urine, containing 100 μg/l 4′-OH-PPC, 6-OH-PPC, 7-OH-PPC and PPC after pretreatment with β-glucuronidase and extraction at a detector sensitivity of 0.01 a.u.f.s.; (c) a urine extract after pretreatment with β-glucuronidase from a patient receiving PPC. Concentrations of free and glucuronidated 4′-OH-PPC, 7-OH-PPC and PPC are 59.5, 48.8 and 121.3 μg/l, respectively, at a detector sensitivity of 0.01 a.u.f.s. Peaks: 1 = 4′-OH-PPC; 2 = 6-OH-PPC; 3 = 7-OH-PPC; 4 = PPC; 5 = endogenous component.

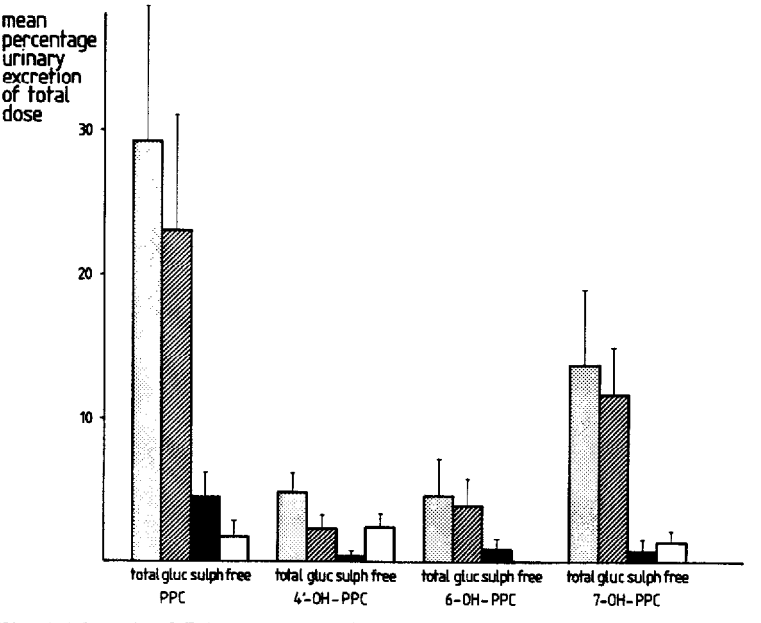


Fig. 4. Mean (± S.D.) percentage urinary excretion of total, glucuronidated, sulphated and free PPC, 4′-OH-PPC, 6-OH-PPC and 7-OH-PPC in twelve patients with a mean daily dosage of 1.3–4.2 mg of PPC.

PPC and PPC, and an extract of a patient's urine on PPC treatment. In spite of several clean-up steps during the extraction of urine a broad "solvent" front proved to be inevitable. Probably this front is due to the presence of related coumarin analogues from food.

Mean (±S.D.) values for the urinary excretion of total, glucuronidated, sulphated and free PPC, 4′-OH-PPC, 6-OH-PPC and 7-OH-PPC (expressed as percentage of the total dosage in these patients) are presented in Fig. 4. It can be concluded that in the conjugation of PPC and its hydroxy metabolites sulphation is a minor step in comparison with glucuronidation. A part of the formed sulphate conjugates is probably hydrolysed in the bladder by endogenous sulphatase, but this part will be small because concentrations of the free products are low.

## CONCLUSION

The separation of PPC and its hydroxy metabolites with an HPLC system is relatively simple. However, analysis of these compounds in urine is complicated by the presence of many interfering endogenous components, which necessitates laborious extraction procedures. The presented method provides the specificity required for the determination of free, glucuronidated and sulphated 4′-OH-PPC, 6-OH-PPC, 7-OH-PPC and PPC in human urine. In this study two metabolites, 5-OH-PPC and 8-OH-PPC, could not be detected in the urine of patients on a daily dosage of 1.3–4.2 mg PPC.

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