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DETERMINATION OF THE ANTICOAGULANT PHENPROCOUMON IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

The determination of the anticoagulant phenprocoumon in plasma, after acidification and extraction with 1,2-dichloroethane was effected through isocratic high-performance liquid chromatography; a C_{18} reversed-phase column was used as stationary phase using aqueous acetonitrile as eluent and UV detection at 313 nm; *p*-chlorophenprocoumon was used as internal standard.

A high proportion of phenprocoumon in urine is eliminated as the glucuronide and must be hydrolyzed enzymatically before extraction; the same column and detector as for plasma were used, but with gradient elution.

The method was used in the range 0.1—5 mg/l, the sensitivity was 0.1 mg/l for plasma and 0.02 mg/l for urine, the precision was in the range 3—5% and the absence of interference due to other anticoagulants, drugs or endogenous compounds allows the specific determination of phenprocoumon in plasma and urine from patients and volunteers in clinical relevant cases, drug interaction, compliance, toxicological and pharmacokinetic studies.

INTRODUCTION

Phenprocoumon (Fig. 1, 1) [4-hydroxy-3-(1-phenylpropyl)-2H-1-benzopyran-2-one] (Marcumar®, Liquamar®), a member of the 4-hydroxycoumarin-type oral anticoagulants [1, 2] is the most commonly used of its type in many countries. This drug, as well as the congeners warfarin (3) and acenocoumarin (4) are used therapeutically for the prophylaxis and treatment of thromboembolic disorders [2, 3]. Coumarin-type anticoagulants inhibit competitively

*Dedicated to Professor Dr. Dr. h.c.mult. G. Schettler on his 65th birthday.

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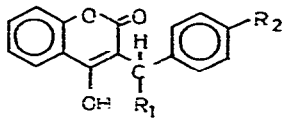

		R_1	R_2
1	Phenprocoumon	$-CH_2.CH_3$	$-H$
2	p—Chlorophenprocoumon	$-CH_2.CH_3$	$-Cl$
3	Warfarin	$-CH_2.CO.CH_3$	$-H$
4	Acenocoumarin	$-CH_2.CO.CH_3$	$-NO_2$

Fig. 1. Structures of 4-hydroxycoumarin anticoagulants.

the last stage of the synthesis of the vitamin K-dependent clotting factors in the liver (carboxylation of glutamic acid to γ-carboxyglutamic acid) [2, 4].

The anticoagulant effect in patients is controlled by the determination of the thromboplastin time [5] or the thrombotest [6] coagulation methods. However, the individual dose of phenprocoumon that leads to effective anticoagulation varies widely with patients [7]. Thus the measurement of phenprocoumon plasma levels and urine excretion rates are indicated particularly during the anticoagulant treatment of patients with oversensitivity or resistance to phenprocoumon, in cases of suspected intoxication or non-compliance and in pharmacokinetic and drug interaction studies [2, 7, 8].

Several methods have been described for the determination of phenprocoumon plasma levels: fluorimetry [9, 10], thin-layer chromatography (TLC) [11—16], gas-liquid chromatography (GLC) [17—22] and high-performance liquid chromatography (HPLC) [16, 23—26]; for the determination of urinary excretion only a GLC method has been reported, after hydrolysis, adsorption and trimethylsilylation, for studies with volunteers after single intravenous injection [19]; however, these methods are inadequate for the analysis of plasma and urine samples from patients, usually under multiple-drug treatment, because they are not specific or sensitive enough, or because they are very time consuming.

In this paper we describe a method for the determination of phenprocoumon in human plasma and urine, involving reversed-phase HPLC after extraction from plasma or enzymatic hydrolysis and extraction from urine, using *p*-chlorophenprocoumon (2) as internal standard.

EXPERIMENTAL

Apparatus

Liquid chromatography was carried out with the following components: Model 6000A pumps, a U6K injector, a Model 660 solvent programmer, a Model 440 UV detector (all from Waters, Königstein, G.F.R.), a BD8 potentiometric recorder (Kipp Analytica, Solingen, G.F.R.), and an Autolab System I computing integrator (Spectra-Physics, Darmstadt, G.F.R.). Centrifugations were run at 2000 *g* at room temperature.

Reagents

All test substances and reagents were of analytical-reagent grade. Acetonitrile (for spectroscopy, Merck, Darmstadt, G.F.R.) and distilled water (B. Braun, Melsungen, G.F.R.) for the mobile phase were used without further purification. Enzymes used for hydrolysis were β-glucuronidase/arylsuphatase from *H.*

pomatia (12 and 60 U/ml) (Merck), β-glucuronidase from *E. coli* (20 U/ml) and arylsulphatase from *H. pomatia* (25 U/ml) (Boehringer Mannheim, Mannheim, G.F.R.). Enzymatic activities were determined using *p*-nitrophenyl-β-D-glucuronide and sulphate as substrates [27]. Phenprocoumon (1) and *p*-chlorophenprocoumon (2) were gifts from Hoffmann-La Roche (Grenzach, G.F.R.) and acenocoumarin (4) and ethyl biscoumacetate from Ciba-Geigy (Wehr, G.F.R.).

Standard solutions

Solutions of 20, 100, 200 and 500 mg/l of phenprocoumon and 500 mg/l of *p*-chlorophenprocoumon (internal standard) in methanol were prepared and kept at 4°C in the dark.

Blood and urine sampling

Venous blood was collected in heparinized tubes, immediately centrifuged, and the plasma kept at −23°C; 24-h urine was stored at the same temperature; samples were thawed and centrifuged before analysis.

Plasma extraction

To 1 ml of plasma contained in a 25-ml centrifuge tube, 10 μl of internal standard solution, 10 μl of methanol, 200 μl of 3 *N* hydrochloric acid and 5 ml of 1,2-dichloroethane were added; the tube was stoppered and shaken for 10 min horizontally at room temperature. After centrifuging for 5 min, 4 ml of the lower phase were transferred into conical centrifuge tubes (15 ml) and evaporated under a stream of nitrogen at 50°C. Before analysis the residue was dissolved in 100 μl of the mobile phase, heated in a water-bath and sonicated. A 20-μl volume of the solution was injected into the chromatograph. A calibration graph was obtained by the same procedure using blank plasma containing 10 μl of the standard solution instead of 10 μl of methanol.

Urine extraction

Enzymatic hydrolysis. A 1-ml volume of urine was treated with 100 μl of acetate buffer (0.1 *M*; pH 4.5) and 10 μl of β-glucuronidase/arylsulphatase and incubated at 37°C for 6 h. Then the mixture was acidified with 200 μl of 3 *N* hydrochloric acid and extracted with 6 ml of 1,2-dichloroethane using the same amount of internal standard and the procedure as for plasma. The corresponding spiked blank urines for the calibration were run in parallel.

Acid hydrolysis. A 1-ml volume of urine was heated with 10 μl of 3 *N* hydrochloric acid at 90°C for 1 h; it was cooled, internal standard was added and the extraction effected as above, omitting the enzymatic hydrolysis step.

Free phenprocoumon. Urine was extracted as described under enzymatic hydrolysis, except that no enzyme or incubation was employed.

Chromatographic conditions

Plasma. The mobile phase was acetonitrile—water—acetic acid (600:400:5), which was filtered (0.45 μm pore size) and degassed before use; the flow-rate was 2.0 ml/min and the pressure 65—100 bar; UV detection was effected at 313 nm at a sensitivity of 0.05 a.u.f.s.; quantitation was effected by area

integration. Calibration graphs were calculated from the linear regression curve from the ratio of the peak area of phenprocoumon to that of the internal standard vs. added phenprocoumon concentration (mg/ml). A reversed-phase C_{18} column was used for the separation (LiChrosorb RP-18 with 10-μm particles, 25 cm × 4 mm I.D.; Hibar RT 250-4; Merck); a guard column (30 × 4 mm I.D.) was filled with Bondapak C_{18} Corasil (35—50-μm particles; Waters) and renewed every few weeks; after finishing a series of analyses the columns were washed with methanol; separations were run at room temperature.

Urine. Urine extracts were analysed using solvent gradient elution with acetonitrile—water—acetic acid (400:600:5) as solvent A and acetonitrile—acetic acid (1000:5) as solvent B; a linear gradient from 0—100% of solvent in 30 min at a total flow-rate of 2.0 ml/min was used. The duration of analysis was 15 min and further 5 min were used for re-equilibration under the initial conditions before the next sample could be injected. Other chromatographic parameters were the same as for plasma.

RESULTS

Plasma

Fig. 2a shows a plasma spiked only with the internal standard; Fig. 2b is from a patient under phenprocoumon treatment and Fig. 2c from a patient with a suspected overdose of phenprocoumon. Retention times were 3.40 and 4.70 min for phenprocoumon and the internal standard, respectively; each sample can be analysed in 5—6 min; peak identity and purity from patient plasma extracts were confirmed by measuring the absorbance ratios for phen-

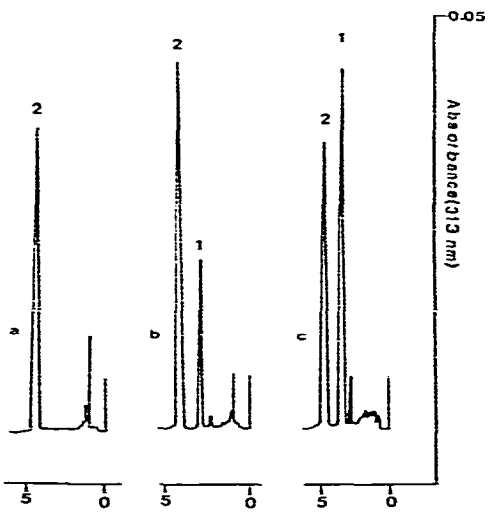

Fig. 2. Chromatograms of plasma extracts: (a) pre-treatment plasma spiked with *p*-chlorophenprocoumon (internal standard; concentration 5 mg/l); (b) extract from a patient under phenprocoumon therapy (phenprocoumon concentration 1.96 mg/l); (c) extract from a patient with suspected phenprocoumon overdosage (concentration 3.8 mg/l). Peaks: 1 = phenprocoumon; 2 = internal standard.

procoumon and internal standards at 280, 254 and 313 nm, which were the same as for the pure compounds. The calculated recoveries for phenprocoumon and *p*-chlorophenprocoumon from plasma were 86.5 ± 3.4% and 88.3 ± 4.7% (n = 5) at a concentration of 5 mg/l; the same values were obtained for aqueous solutions. Plasma analysis were in the range 0.1—5 mg/l with a linear calibration regression line of $y = 0.2188x + 0.0125$, $r = 0.998$; sensitivity 0.1 mg/l.

Urine

Fig. 3a shows the separation of phenprocoumon and internal standard employing the linear gradient elution conditions used for urine analysis (the retention times were phenprocoumon 11.02 min and *p*-chlorophenprocoumon 13.29 min). Fig. 3b shows the chromatogram of a blank urine and Fig. 3c that of a blank urine spiked with 0.2 mg/l of phenprocoumon and 5 mg/l of internal standard. Fig. 4 shows chromatograms of the urine of a patient under chronic phenprocoumon treatment: (a) direct extraction with no hydrolysis, (b) after acid hydrolysis and (c) after enzymatic hydrolysis. The analysis time was 20 min per sample; peaks were identified in a similar way as for plasma. Calculated recoveries at a concentration of 5 mg/l for phenprocoumon and *p*-chlorophenprocoumon were 88.9 ± 2.7% and 89.1 ± 2.2%, respectively (n = 5); analyses were in the range 0.02—5 mg/l with a linear regression line for the calibration of $y = 0.2189x + 0.0189$, $r = 0.998$; sensitivity, 0.02 mg/l. The accuracy and precision for plasma and urine are given in Table I.

Validity

Plasma levels from ten orally anticoagulated patients (dose: 2.33 ± 0.64 mg/day; mean ± standard deviation) showed a mean concentration of 2.10 ± 0.66 mg/l of total phenprocoumon (range 1.54—3.58 mg/l). Urine samples from another group of anticoagulated patients were analysed and the results are shown in Table II; the mean values for the 24-h phenprocoumon excretion were 0.0412 mg for free phenprocoumon and 0.388 mg for total phenprocoumon after enzymatic hydrolysis. The values obtained after hydrolysis with acids were always lower than those obtained with enzymes; the concentraprocoumon after enzymatic hydrolysis. The values obtained after hydrolysis with acids were always lower than those obtained with enzymes; the concentration ratio of enzymatic to acid hydrolysis was 2.19 ± 0.66 (n = 10). The method was also used for the analysis of plasma and urine samples from patients with oversensitivity to anticoagulants, suspected non-compliance or overdose, and for drug interaction studies [2, 8].

Interferences

There were no interferences in the assay from plasma and urine from patients treated with following drugs: allopurinol, atenolol, cimetidine, carbamazepine, digoxin, digitoxin, hydrochlorothiazide, indomethacin, isosorbide dinitrate, β-methyldigoxin, nifedipine, nomifensin, pindolol and triamterene. The following substances were extracted and did not interfere: acenocoumarin, acetaminophen, β-acetyldigoxin, acetylsalicylic acid, ampicillin, atropine, azlocillin, caffeine, cefazolin, cotinine, dihydralazine, ergotamine, ethyl biscoumacetate, furosemide, heparin, heptabarbital,

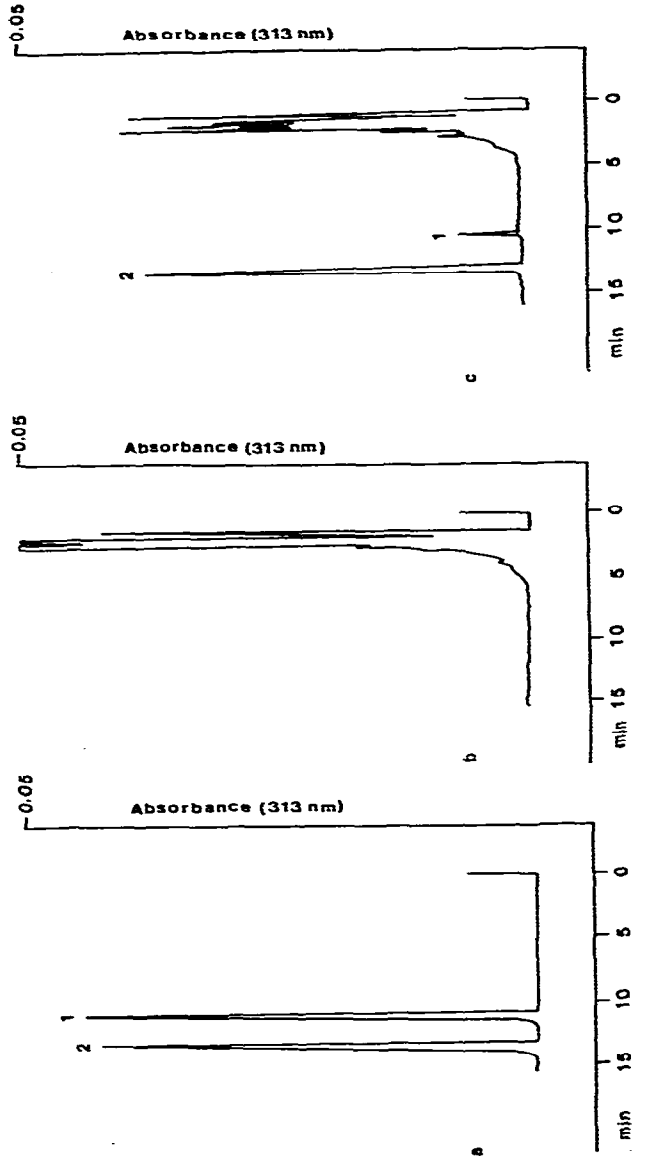

Fig. 3. Chromatograms of urine extracts: (a) separation of pure phenprocoumon and *p*-chlorophenprocoumon; (b) pre-treatment urine with no addition of internal standard; (c) extract from urine spiked with 0.2 mg/l of phenprocoumon and 5 mg/l of internal standard. Peaks as in Fig. 2.

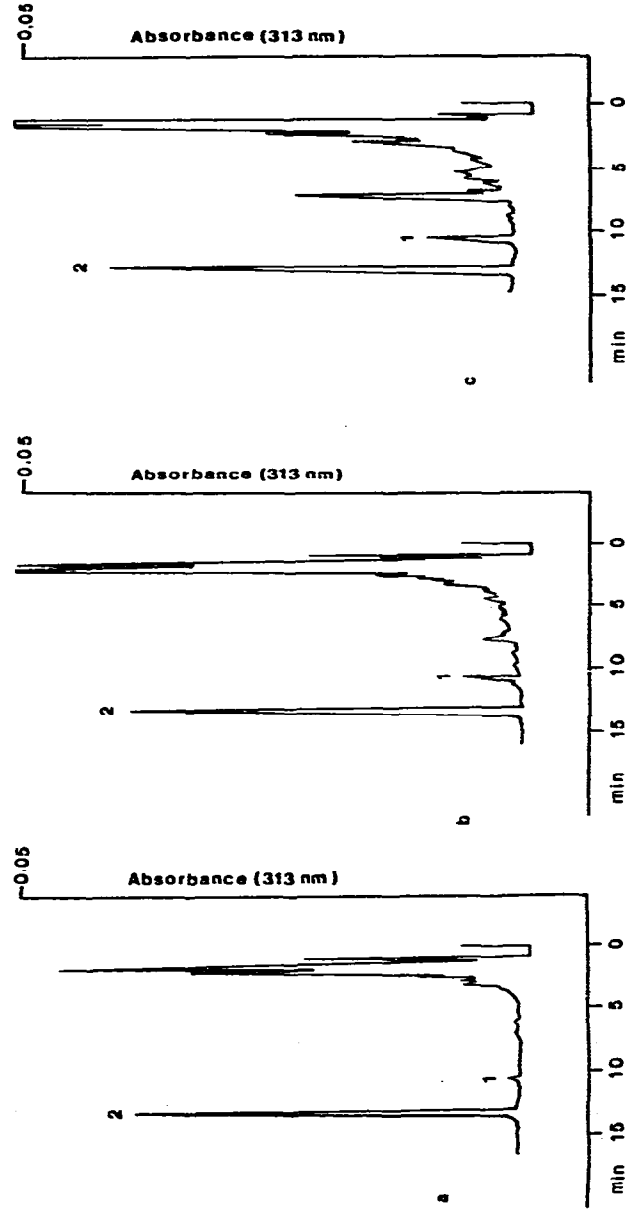

Fig. 4. Chromatograms from the urine of a patient under phenprocoumon treatment: (a) extract with no previous hydrolysis (phenprocoumon concentration 0.02 mg/l); (b) extract after acid hydrolysis (concentration 0.19 mg/l); (c) extract after enzymatic hydrolysis (concentration 0.35 mg/l). In all instances the concentration of the internal standard was 5 mg/l. Peaks as in Fig. 2.

TABLE I

REPRODUCIBILITY OF PLASMA AND URINE ANALYSES FOR PHENPROCOUMON

Parameter	Plasma				Urine		
Concentration given (mg/l)	0.5	1	2	5	0.2	1	5
Concentration found (mg/l)	0.52	1.03	2.03	4.98	0.22	0.96	5.01
Coefficient of variation (%) (n=5)	6.1	5.6	5.0	3.5	2.2	5.3	2.2

TABLE II

PHENPROCOUMON CONCENTRATION IN URINE AND 24-h EXCRETION RATE IN PATIENTS ON PHENPROCOUMON MAINTENANCE THERAPY (2.33 ± 2.82 mg/day; n = 9)

The upper values are mean ± standard deviation; the values in parentheses are the ranges.

Phenprocoumon	Concentration (mg/l)	24-h excretion (mg)
Free	0.0267 ± 0.0245 (0—0.07)	0.0412 ± 0.0267 (0—0.13)
After enzyme hydrolysis	0.466 ± 0.428 (0.10—1.46)	0.388 ± 0.236 (0.173—0.936)

hippuric acid, 1-(β-hydroxypropyl)theobromine, isoproterenol, lidocaine, metoprolol, methaqualone, mezlocillin, nicotine, neostigmine bromide, penicillin G, phenacetin, phenobarbital, pindolol, procainamde, propranolol, quinidine, salicylic acid, salicylamide, secobarbital, sulphinpyrazone, theobromine, theophilline, trithiozine, uric acid, vitamin B complex, vitamin C and warfarin.

DISCUSSION

Plasma

Several procedures for the pre-cleaning of phenprocoumon in plasma were tried: precipitation of proteins with solvents [23], prepurification through C-18 cartridges [28] and adsorption [26]; they were found to be non-reproducible or the chromatograms showed interfering peaks; 1,2-dichloroethane extraction in acidic media was found to be the most reproducible method of several tested, and also showed good recoveries and absence of interferences. A wavelength of 313 nm was selected for better selectivity to avoid the detection of substances that would interfere at lower wavelengths. The method measures total phenprocoumon concentration; it has been applied in the cases mentioned above under *Validity* and has been in current use for over a year.

Urine

Only a small fraction (1.8%) of the applied phenprocoumon dose is eliminated unchanged in urine (Table II) and sometimes could not be detected with the described method. In rats, phenprocoumon is metabolized by liver

microsomes through oxidation to the 4'-, 6-, 7- and 8-monohydroxy derivatives [12, 13, 29]; in man, after single intravenous application of phenprocoumon a high proportion is eliminated as the glucuronide [19] (probably conjugated glycosidically to the 4-hydroxy group of phenprocoumon). Prior to extraction, urine was submitted to hydrolysis to liberate phenprocoumon, which was later extracted and quantitated by HPLC. The complex nature of urine samples, due to interfering endogenous compounds, other drugs and metabolites, necessitates the use of solvent gradient elution during the HPLC run. The conditions for hydrolysis described under Experimental were those selected for maximum phenprocoumon after several optimization trials; acid hydrolysis always yielded lower values than enzymatic hydrolysis (see *Validity*), owing in part to the degradation of phenprocoumon at low pH and high temperature; hydrolysis with β-glucuronidase/arylsuphatase gave the same value as with β-glucuronidase alone, confirming the observation that phenprocoumon is not conjugated with sulphates [19].

Excretion of phenprocoumon was studied in 24-h urine samples obtained from hospitalized patients; free phenprocoumon accounts for 1.8% of the applied dose, and after enzymatic hydrolysis 16.6% (Table II); inter-individual differences are present owing to variable dose, different multiple drug treatments and different disorders.

A suspected case of phenprocoumon overdose showed a 24-h elimination of total phenprocoumon (after enzymatic hydrolysis) of 4.6 mg (and a plasma level of 3.80 mg/l; Fig. 2c), significantly higher than the mean and extremes found for patients under normal phenprocoumon therapy, confirming the suspicion of intoxication.

In conclusion, free phenprocoumon or phenprocoumon after acid hydrolysis is not a reliable indicator of urine elimination; values obtained after enzymatic hydrolysis are mor trustworhty in this sense.

For the reasons expressed above the method is simple, rapid, sensitive, specific and reproducible for the quantitation of phenprocoumon in plasma and urine from patients and volunteers in clinical cases, drug interaction, compliance, toxicological and pharmacokinetic studies.

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