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Note

Determination of the plasma protein binding of the coumarin anticoagulants phenprocoumon and its metabolites, warfarin and acenocoumarol, by ultrafiltration and high-performance liquid chromatography

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Recent studies of the biotransformation of the oral anticoagulant phenprocoumon (PH) (Fig. 1) (Marcumar®) in humans showed that this drug is metabolized by oxidation to 7-, 6- and 4′-hydroxyphenprocoumon (Fig. 1) [1–4].

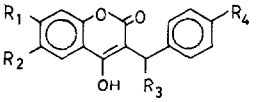

Fig. 1. Molecular structures of coumarin anticoagulants.

	R_1	R_2	R_3	R_4
Phenprocoumon	H	H	C_2H_5	H
7-Hydroxyphenprocoumon	OH	H	C_2H_5	H
6-Hydroxyphenprocoumon	H	OH	C_2H_5	H
4′-Hydroxyphenprocoumon	H	H	C_2H_5	OH
Warfarin	H	H	CH_2COCH_3	H
Acenocoumarol	H	H	CH_2COCH_3	NO_2

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The metabolites also are present with the drug in plasma [5] and are eliminated in urine [2–4] and bile [6].

Since only unbound substances can be metabolized by the liver, it is important to measure the plasma protein binding of these compounds; diseases, age, drug interactions and other individual factors may alter the extent of binding [7–10].

PH protein binding has been measured by equilibrium dialysis followed by fluorimetry [11], by scintillation counting of ^{3}H-labelled PH [12] or by continuous ultrafiltration with UV detection [13]. Unbound fraction values [7] ($f_u = 100 \times$ unbound drug concentration/total drug concentration) of 0.2–0.8% have been reported [11–13]. However, no information is available about the plasma protein binding of the PH metabolites and its possible influence on the binding of the parent drug.

The aim of our investigation was to develop a simple, fast, sensitive and accurate assay that would not require labelled compounds for the determination of the plasma protein binding capacity of PH and its metabolites in samples obtained from healthy individuals and from patients with different disease states. PH as racemate (the therapeutically used drug), its enantiomers and the 7-, 6- and 4′-hydroxy-PH metabolites were analysed. The method was extended to the measurement of the protein binding of the coumarin anticoagulant drugs warfarin and acenocoumarol (Fig. 1).

EXPERIMENTAL

Plasma samples

Venous blood from healthy volunteers was collected in heparinized tubes and centrifuged; plasma albumin levels were normal; plasma samples were kept at −20°C and thawed and centrifuged before analysis. Samples for clinical studies were obtained as detailed elsewhere [14]. The same plasma samples from ten different individuals were used for the analysis of (*S*)- and (*R*)-PH. Plasma samples for the analysis of racemic PH and metabolites were from different individuals than those for the pure enantiomers.

Chemicals

Reagents were analytical grade. Methanol and acetonitrile were spectroscopic grade (Merck, Darmstadt, F.R.G.). PH and its 7-, 6- and 4′-hydroxy metabolites were synthesized in our laboratories [15]. PH enantiomers and *p*-chlorophenprocoumon (*p*-Cl-PH) were gifts from Hofmann-LaRoche (Grenzach-Wyhlen, F.R.G.) and acenocoumarol from Ciba-Geigy (Wehr, F.R.G.). Warfarin was commercially available (Sigma-Chemie, Deisenhofen, F.R.G.).

Solutions

Stock solutions of PH and its metabolites (1 mg/ml) were prepared in 0.1 *M* phosphate buffer (pH 7.4). Standard solutions were obtained by dilution with the same buffer and were kept at 4°C in the dark.

Ultrafiltration

A 0.5-ml volume of plasma was incubated for 1 h with 5 μg of PH and then transferred to a Centricon 30 000 tube (Amicon, Witten, F.R.G., molecular weight cut-off 30 000) and centrifuged at 3900 *g* at room temperature for 30 min (temperature-controlled centrifuge J6B, Beckman Instruments, Munich, F.R.G.). A 150-μl volume of the ultrafiltrate was mixed with 5 μg of *p*-Cl-PH (internal standard), and 150 μl were injected into the HPLC system.

The same procedure was followed for the PH enantiomers and the metabolites, warfarin and acenocoumarol.

To examine the displacing effects of PH and its metabolites, 0.5 ml of plasma was incubated with 5 μg of PH and 5 μg of one of the metabolites and submitted to ultrafiltration in the same way.

Equilibrium dialysis

To 1 ml of plasma, equivalent amounts of PH were added as described above, transferred to the dialysis cell and dialysed against 1 ml of buffer (0.1 *M* phosphate, pH 7.4). The PTFE dialysis cells (Dianorm® system; Bachofer, Reutlingen, F.R.G.) were separated with a membrane (Spectrapor® cellulose membrane, molecular weight cut-off 12 000–14 000, Spectrum Medical Industries, Los Angeles, CA, U.S.A.). The cells were rotated at 15 rpm for 4 h at 37°C in a water-bath, 5 μg of the internal standard were added to 150 μl of the buffer solution, and 150 μl were injected into the HPLC system. For calibration, samples with buffer instead plasma were run simultaneously.

HPLC conditions

The HPLC equipment and fluorescence detector were the same as described previously [1,2]. System A consisted of a LiChrosorb RP18 column (126 mm × 4 mm I.D., 7 μm particle size, Merck), used with isocratic elution and the same guard column and solvent as previously described [1]. The ultrafiltrates from PH, warfarin and acenocoumarol were analysed with this system ($k' = 3.07$, 3.03 and 2.86, respectively). System B consisted of the same column, solvents and elution gradient as described before [2], and was used to analyse the samples from 7-, 6- and 4′-hydroxy-PH ($k' = 5.05$, 3.61 and 2.85, respectively).

Calculations

The protein binding was calculated by comparing the peak-area ratios of PH and the internal standard from plasma sample ultrafiltrates with those of an ultrafiltrate with buffer containing the same amount of drug, but no protein

(100% unbound). The ratios were linearly proportional to the concentrations in the measured range.

RESULTS AND DISCUSSION

Phenprocoumon

Human plasma protein binding data of PH, its metabolites and other coumarin anticoagulants obtained by ultrafiltration are shown in Table I. They show the mean f_u (f_u = % bound) values obtained from samples of ten different subjects who had normal albumin levels.

Racemic (*RS*)-phenprocoumon showed a strong plasma protein binding, confirming previous reports [11–13]. The limit of detection was 20 ng/ml, and a precision and accuracy of ±5% were obtained.

HPLC analysis of the PH drug calibration solution is shown in Fig. 2a. Fig. 2b is from a plasma sample after incubation with PH, ultrafiltration and HPLC.

To validate the ultrafiltration method the binding data for PH were compared with those obtained by equilibrium dialysis and shown to be equivalent. More reliable data were obtained by ultrafiltration owing to the absence of disturbing factors (i.e. volume shifts, presence of buffer) [16,17]; other advantages of ultrafiltration compared with equilibrium dialysis [18] are the simpler analytical handling and shorter analysis times (30 min for filtration versus 4 h for dialysis).

The use of non-radioactive drug means easier handling and ready access to the compounds being investigated (labelled compounds are not readily available or synthesized). Moreover, plasma protein binding studies by Mungall et al. [19] for the analogous coumarin anticoagulant warfarin showed that more reliable values were obtained using the non-labelled compound with equilibrium dialysis and HPLC, than when the radioactive tracer technique was used.

TABLE I

PLASMA PROTEIN BINDING OF PHENPROCOUMON, ITS METABOLITES, WARFARIN AND ACENOCOUMAROL DETERMINED BY ULTRAFILTRATION AND HPLC

Plasma samples from ten different subjects; $n = 10$.

Compound	Percentage unbound (mean ± S.D.)
Phenprocoumon	0.390 ± 0.029
(*S*)-Phenprocoumon	0.198 ± 0.024
(*R*)-Phenprocoumon	0.322 ± 0.019
7-Hydroxyphenprocoumon	0.980 ± 0.235
6-Hydroxyphenprocoumon	0.895 ± 0.232
4′-Hydroxyphenprocoumon	0.636 ± 0.233
Warfarin	0.499 ± 0.121
Acenocoumarol	1.164 ± 0.165

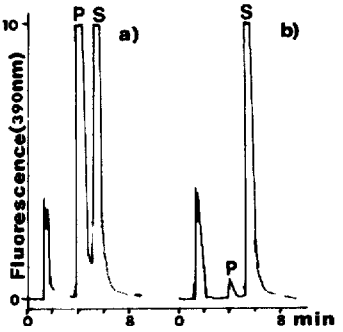

Fig. 2. Chromatograms of (a) buffer calibration solution (10 μg/ml) and (b) plasma, after incubation with PH and ultrafiltration. Peaks: P = PH; S = internal standard.

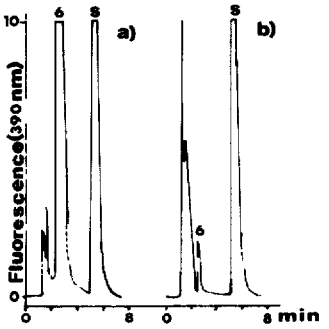

Fig. 3. Chromatograms of (a) buffer calibration solution (10 μg/ml) and (b) plasma, after incubation with 6-hydroxy-PH and ultrafiltration. Peaks: 6 = 6-hydroxy-PH; S = internal standard.

To examine a possible displacing effect of other substances on PH bound to plasma proteins, the binding of the drug was also determined in the presence of each metabolite. HPLC peaks from PH and metabolites were well separated, as reported previously [2]. Similar f_u data were obtained for the PH binding in the absence or presence of each metabolite, indicating that no significant displacement of PH from protein binding sites by the metabolites occurred.

The plasma protein binding was examined using PH concentrations in the range 2–10 μg/ml of plasma, and the same f_u values were obtained. The higher concentrations were chosen for the assay because of better accuracy and precision.

No interfering peaks were detected during analysis.

This method was used to study the influence of maintainance haemodialysis on PH plasma protein binding in patients [14].

The f_u from the single (*S*)- and (*R*)-enantiomers of PH differed significantly (Table I) as was shown previously by other workers [20]; this fact may

play a role in the stereoselective elimination of PH [20–22]. A lower mean f_u was obtained for the plasma binding of (*S*)- and (*R*)-PH than for that of the racemate (Table I). Samples from different groups of individuals (see Experimental) and intra-individual differences cause this discrepancy.

Phenprocoumon metabolites

The protein binding of the individual PH metabolites is shown in Table I. Analysis of the 6-hydroxy-PH calibration solution is shown in Fig. 3a and, plasma after incubation with 6-hydroxy-PH, ultrafiltration and HPLC, in Fig. 3b. Although a high degree of binding was found for these compounds, they did not displace PH from plasma proteins, as mentioned above.

Warfarin and acenocoumarol

The protein binding of warfarin and acenocoumarol was determined with the same techniques as for PH (Table I). Protein binding values were comparable with those in the literature [23, 24].

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