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SEPARATION OF THE ENANTIOMERS OF PHENPROCOUMON AND WARFARIN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING A CHIRAL STATIONARY PHASE

DETERMINATION OF THE ENANTIOMERIC RATIO OF PHENPROCOUMON IN HUMAN PLASMA AND URINE

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SUMMARY

The enantiomers of the chiral coumarin-type anticoagulants phenprocoumon, warfarin and *p*-chlorophenprocoumon were separated by high-performance liquid chromatography on a chiral stationary phase (Nucleosil-Chiral 2®) and normal-phase conditions. Chromatographic peak identification was performed with authentic reference compounds of the enantiomers and on-line UV spectra comparison. This method was applied to the determination of the enantiomeric ratio of phenprocoumon in plasma and urine extracts from patients under racemic drug therapy. The limit of detection (50 and 80 ng/ml) and precision (less than 5%) of the method are adequate for pharmacokinetic and enantioselective disposition studies, respectively, of phenprocoumon. No racemization was detected during the extraction procedures.

INTRODUCTION

The coumarin-type anticoagulants phenprocoumon (PH), warfarin (WA) and acenocoumarol (AC) (see Fig. 1) are widely used for prophylaxis of various thromboembolic disorders and are clinically administered as racemic mixtures [1]. However, the enantiomers differ in their pharmacokinetics [2–9] and show distinct pharmacodynamic responses in humans [2–7].

The enantiomers of PH show different anticoagulant activities in humans; thus the *S*-enantiomer of PH is 1.5–2.5 times more active than the *R*-enan-

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Fig. 1. Structures of coumarin-type anticoagulants (left) and absolute configuration of (*S*)-phenprocoumon (right).

Compound	R_1	R_2
Phenprocoumon (PH)	C_2H_5	H
p-Chlorophenprocoumon (p-Cl-PH)	C_2H_5	Cl
Warfarin (WA)	CH_2COCH_3	H
Acenocoumarol (AC)	CH_2COCH_3	NO_2

tiomer[2]. Pharmacokinetic differences in the behaviour of the enantiomers of PH have been reported, such as different protein binding, clearances, plasma elimination half-lives, volumes of distribution and enantiomeric ratios [2–9].

Studies of the enantioselective pharmacokinetic effects have been carried out by analysing the drugs (and sometimes also the metabolites) after administration of (a) each enantiomer separately [2–7] or (b) pseudoracemates [8,10,11] (equimolar mixtures of (*R*)-^{12}C and (*S*)-^{13}C enantiomers).

Studies of the elimination of PH enantiomers after single-dose administration to healthy subjects were reported [2,8,9]. No data are available about the intra- and inter-individual variabilities or about the influence of other drugs or disease states on the stereoselective kinetics of PH in patients on long-term PH therapy.

The aims of this work were to develop a direct method for the separation of the enantiomers of PH and other anticoagulants using chiral stationary phases and to apply this method for the determination of the enantiomeric ratio of phenprocoumon in human plasma and urine; these data would complete investigations in the disposition of PH [12–16] in man. Using a Nucleosil Chiral-2® stationary phase it was possible to separate the enantiomers of PH and WA by high-performance liquid chromatography (HPLC); this method is currently applied to the determination of the enantiomers of PH in clinical pharmacological studies.

EXPERIMENTAL

Chemicals

Racemic PH, (*R*)-PH and (*S*)-PH, racemic *p*-chlorophenprocoumon (p-Cl-PH), (*S*)-WA and (*S*)-AC were gifts from Hoffmann-LaRoche (Basle, Switzerland) and Dr. H.H.W. Thijssen (Department of Pharmacology, Rijksuniversiteit Limburg, Limburg, The Netherlands); racemic AC was a gift from Ciba Geigy (Grenzach, F.R.G.) and racemic WA was purchased from Sigma Chemie (Deisenhofen, F.R.G.). HPLC solvents were spectroscopic grade.

Solutions

Standard solutions of the enantiomers were prepared in (a) 0.1 *M* phosphate buffer (pH 7.4) or (b) in eluent 1 or 2 (all 1 mg/ml). They were kept at 4°C in the dark and were stable for several weeks.

HPLC enantiomeric separation of pure racemates

The separation of the enantiomers of PH, WA and p-Cl-PH were performed with HPLC systems 1 and 2. System 1 contained a Model 1090M liquid chromatograph with a ternary solvent delivery system, autosampler, photodiode array detector (DAD) and a Model 79994A computer workstation for system control and data handling (Hewlett-Packard, Waldbronn, F.R.G.). The DAD wavelengths were set at 310 and 276 nm; on-line spectra were obtained in the range 200–400 nm. The enantiomers were separated with a Nucleosil Chiral-2 column (250 mm×4 mm I.D., 5 μm particle size; Macherey-Nagel, F.R.G.). Eluent 1 was *n*-heptane–dioxane–trifluoroacetic acid (100:25:0.1, v/v) and eluent 2 was *n*-heptane–dioxane–trifluoroacetic acid (100:30:0.1, v/v). The flow-rate was 1 ml/min and the operating column pressure was 100 bar.

System 2 consisted of a pump (Model M 6000A), an autosampler (WISP 710B) (Waters, Eschborn, F.R.G.), a fluorescence detector set at 310 nm for excitation and 360 nm for emission (SFM 23, Kontron, Eching, F.R.G.) and a plotter–integrator Model CR1-B (Shimadzu, Düsseldorf, F.R.G.). The same column and eluents were used as described in system 1. The enantiomeric ratios of PH, WA and p-Cl-PH were calculated from the peak-area ratios *S*-enantiomer/*R*-enantiomer.

Enantioselective analysis of PH in plasma and urine samples

Venous blood samples from patients during or after long-term PH therapy were obtained, transferred to heparinized tubes, centrifuged immediately and stored at −20°C till analysis. Urine samples were collected and immediately frozen at −20°C. Samples were thawed at 37°C and centrifuged before analysis.

Plasma samples (1 ml) were extracted with 6 ml of dichloroethane after acidification with 100 μl of 3 *M* hydrochloric acid as previously described [12].

Urine samples (10 ml) were incubated for 12 h with 500 μl of 1 *M* acetate buffer and 50 μl of β-glucuronidase/arylsulphatase (Merck, Darmstadt, F.R.G.), and the solution was loaded into a pretreated Sep-Pak C_{18} micro-column (Millipore, Eschborn, F.R.G.). The pre-column was washed successively with 5 ml of 1% aqueous acetic acid, 5 ml of methanol–1% aqueous acetic acid (40:60) and finally with 4 ml of methanol. The methanolic solution was filtered through a Sep-Pak Silica micro-column and evaporated under nitrogen.

The evaporated plasma extracts were redissolved in 100 μl of eluent 1, and 80 μl were injected into HPLC system 2.

The extracted residues from urine samples were redissolved in 40 μl of eluent, and 25 μl were injected into HPLC system 1.

The enantiomeric ratios of PH from plasma and urine extracts were obtained using the peak-area ratio (*S*)-PH/(*R*)-PH. These ratios were calculated as follows: mixtures of the pure enantiomers with enantiomeric ratios 0.5, 1.0 and 2.0 and a total PH concentration of 2.0 μg/ml were added to blank plasma and urine, extracted and analysed; a linear calibration curve for peak-area ratios and enantiomeric ratios was obtained.

Analysis of total PH concentrations in plasma and urine

The determination of racemic PH in plasma and urine extracts was performed with HPLC using gradient elution, a C_{18} reversed-phase column and fluorescence detection as previously described [12,13]; p-Cl-PH was used as internal standard. Linear calibration curves for total PH concentrations in plasma and urine were obtained in the ranges 0.1–4.0 and 0.05–2.0 μg/ml for plasma and urine, respectively.

The same plasma sample was analysed independently for the determination of the total PH concentration and the enantiomeric ratio. The concentrations of the individual (*S*)-PH and (*R*)-PH enantiomers were calculated from the enantiomeric ratio and the total PH concentration data.

RESULTS AND DISCUSSION

A great number of drugs are chiral or prochiral and show stereoselective effects in biological systems [17–19]. The absolute configuration of the chiral centre of the coumarin-type anticoagulant enantiomers was determined for each drug with chemical and chiroptical methods [20–23]. Although pharmacokinetic studies of the separation of the optical antipodes of WA and AC in biological fluids using chiral derivatizing agents [24–26] or chiral stationary phases [27] have been published, no reports are available about methods for the separation of PH enantiomers and measurement of the stereochemical effects of PH after administration of the racemate.

In recent years several chiral phases for the chromatographic separation of mixtures of enantiomers have been developed [28–32]. As seen above, the use of one of these chiral stationary phases (Nucleosil Chiral-2) allowed the separation of the enantiomers of PH and WA. According to the manufacturers (Dr. I. Sebestian, personal communication) it consists of L-phenylethylamine covalently bound through its nitrogen to a 2,4-dinitrobenzyl group and L(+)-tartaric acid; the latter is bound to the silica support through an amide group.

Separation of the enantiomers of pure PH, WA and p-Cl-PH

The chiral column resolved racemic PH and p-Cl-PH using system 1 and eluent 1 (see Fig. 2a, p-Cl-PH chromatogram not shown); racemic WA was resolved using system 2 and eluent 2 (see Fig. 2b).

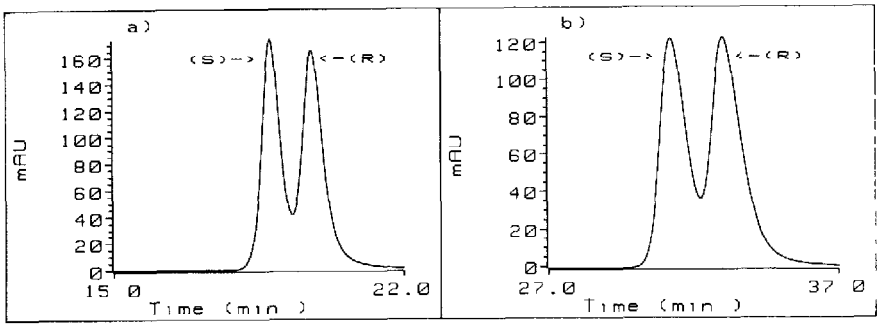

Fig. 2. HPLC enantioseparations of (a) (*R,S*)-phenprocoumon (eluent 1) and (b) (*R,S*)-warfarin (eluent 2). For conditions see Experimental.

TABLE I

ENANTIOSEPARATION DATA OF PURE DRUGS

Substance	Retention time (min)		Separation factor
	(*S*)	(*R*)	
PH	18.77	19.77	1.062
WA	31.22	33.02	1.062
p-Cl-PH	18.82	19.82	1.062

The enantiomers were identified by HPLC with reference compounds (Table I) and on-line UV spectra.

Racemic AC was not resolved even after numerous modifications of the eluent composition.

The analysis of racemic PH showed a precision ($n=10$) of $\pm 1.2\%$ and $\pm 0.72\%$ for the individual area under the curve (AUC) of each PH enantiomer; a precision of $\pm 0.51\%$ was obtained for the enantiomeric ratio.

Mixtures of enantiomers containing 1% *S*-enantiomer are still chromatographically resolvable; however, mixtures containing less than 10% *R*-enantiomer were not separated by liquid chromatography.

This method allows the direct measurement of the enantiomeric ratio of PH and WA without resort to reactions with chiral derivatizing agents, as reported for WA and AC [24–26].

This method may also be applied to the control of the optical purity of enantiomers of PH and WA or their mixtures with the limitations mentioned above and to the microscale separations of pure enantiomers from racemic mixtures without long fractional crystallization of diastereomeric salts.

Enantioselective HPLC bioassay of PH in humans

We applied the chiral phase method for the separation of pH enantiomers by HPLC and the determination of the enantiomeric ratio of PH in plasma and urine samples. Although no baseline chromatographic separation of the enantiomers was achieved (see Fig. 3), the method with the chiral column was adequate for the analysis of PH enantiomers. Chromatograms from a patient's plasma and urine extracts are shown in Fig. 3c and d. Fig. 4 shows the plasma concentration–time curve for the enantiomers of PH in a patient after therapy interruption.

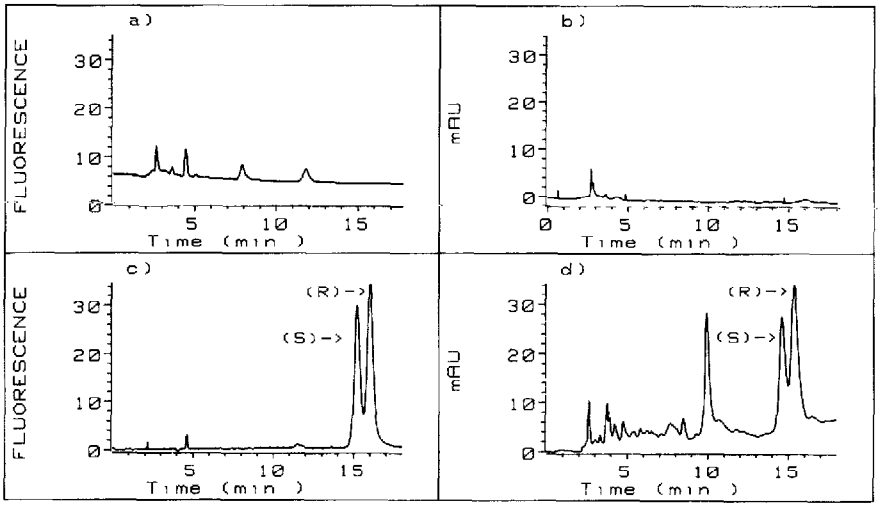

Fig. 3. Chromatograms of biological samples using the chiral phase: (a) and (b) blank plasma and urine extracts; (c) and (d) plasma and urine extracts from a patient under PH therapy.

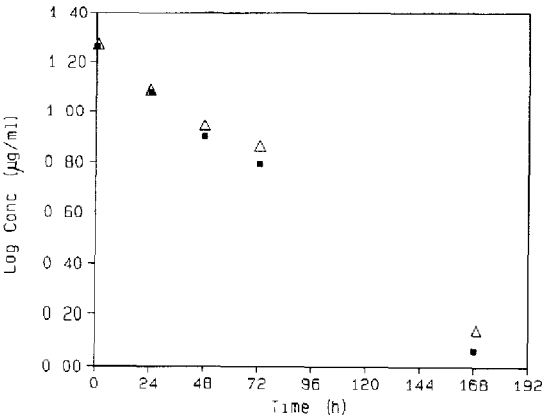

Fig. 4. PH enantiomers plasma concentration–time curves of a patient after PH therapy interruption. (■) (*S*)-PH; (△) (*R*)-PH.

The limits of detection for plasma and urine extracts were 80 ng/ml for each enantiomer with DAD and 50 ng/ml with fluorescence detection. The precision for the enantiomeric ratio analysis of PH in plasma and urine was $<5\%$ ($n=11$).

The method was validated because (a) blank plasma and urine extracts did not shown interfering chromatographic peaks (Fig. 3a and b); (b) numerous drugs [12] did not interfere with the test; (c) no racemization of PH during extraction and analysis of the PH enantiomers was detected using blank plasma and urine after addition of the pure enantiomers; (d) the enantiomers were identified by HPLC with reference compounds and on-line UV spectra; (e) linear calibration curves were obtained by analysing blank plasma and urine containing mixtures of enantiomers with an enantiomeric ratio between 0.5 and 2.0.

Racemic p-Cl-PH was used as internal standard for the assay of total PH in plasma; however, it could not be used as such for enantiomeric analysis of biological extracts, because of its coelution with PH enantiomers.

This method could also serve as a basis for the development of an assay for the measurement of the enantiomeric ratio of WA enantiomers in biological fluids.

The HPLC bioassay of PH is being applied to clinical pharmacological studies on the stereochemistry of PH disposition and drug interactions in patients; the results of these investigations will be published elsewhere.

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REFERENCES

1 R.A. O'Reilly, in A.S. Gilman, L.S. Goodman and T.W. Rall (Editors), The Pharmacological Basis of Therapeutics, Macmillan, New York, 7th ed., 1985, p. 1338.
2 E. Jähnchen, Th. Meinertz, H.J. Gilfrich, U. Groth and A. Martini, Clin. Pharmacol. Ther., 3 (1976) 342.
3 J.M. Eble, B.D. West and K.P. Link, Biochem. Pharmacol., 15 (1966) 1003
4 R.J. Lewis, W.F. Trager, K.K. Chan, A. Breckenridge, M. Orme, M. Rowland and W. Schary, J. Clin. Invest., 53 (1974) 1607.
5 J. Gobdillon, J. Richard, A. Gerardin, T. Meinertz, W. Kasper and E. Jähnchen, Br. J. Clin. Pharmacol., 12 (1981) 621.
6 T. Meinertz, W. Kasper, C. Kahl and E. Jähnchen, Br. J. Clin. Pharmacol., 5 (1978) 187.

7 H.H.W. Thijssen, G.M.J. Janssen and L.G.M. Baars, Eur. J. Clin. Pharmacol., 30 (1986) 619.
8 S. Toon, L.D. Heimark, W.F. Trager and R.A. O'Reilly, J. Pharm. Sci., 74 (1985) 1037.
9 D.S. Hewick and A.M. Shepherd, J. Pharm. Pharmacol., 28 (1976) 257.
10 S. Toon, L.K. Low and M. Gibaldi, Clin. Pharmacol. Ther., 39 (1986) 15.
11 W.N. Howald, E.D. Bush, W.F. Trager, R.A. O'Reilly and C.H. Motley, Biomed. Mass Spectrom., 7 (1980) 35.
12 J.X. de Vries, J. Harenberg, E. Weber, R. Zimmermann and M. Simon, J. Chromatogr., 231 (1982) 83.
13 J.X. de Vries, M. Simon, R. Zimmermann and J. Harenberg, J. Chromatogr., 338 (1985) 325.
14 J.X. de Vries, R. Zimmermann and J. Harenberg, Eur. J. Clin. Pharmacol., 29 (1986) 591
15 J.X. de Vries, R. Raedsch, U. Völker, I. Walter-Sack and E. Weber, Eur. J. Clin. Pharmacol., 35 (1988) 433.
16 J.X. de Vries, Chromatographia, 22 (1986) 421.
17 E.J. Ariens, Eur. J. Clin. Pharmacol., 26 (1984) 663.
18 K. Williams and E. Lee, Drugs, 30 (1985) 333.
19 B. Testa, Trends Pharmacol. Sci., (1986) 60.
20 B.D. West and K.P. Link, J. Heterocyclic Chem., 2 (1965) 93.
21 B.D. West, S. Preis, C.H. Schroeder and K.P. Link, J. Am. Chem. Soc., 83 (1961) 2676.
22 L.R. Wheeler and W.F. Trager, J. Med. Chem., 22 (1979) 1122.
23 E.J. Valente and W.F. Trager, J. Med. Chem., 21 (1978) 141.
24 C.R. Banfield and M. Rowland, J. Pharm. Sci., 73 (1984) 1392.
25 T.S. Gill, K.J. Hopkins and M. Rowland, Br. J. Clin. Pharmacol., 25 (1988) 591.
26 G.L. Jeyaraj and W.R. Porter, J. Chromatogr., 315 (1984) 378.
27 S. Allenmark, J. Liq. Chromatogr., 9 (1986) 425.
28 V. Schurig, Angew. Chem., 96 (1984) 733.
29 W.A. König, The Practice of Enantiomer Separation by Capillary Gas Chromatography, Hüthig, Heidelberg, 1987.
30 S. Hara and J. Cazes, J. Liq. Chromatogr., 9 (1986) 241.
31 W.H. Pirkle, in J.C. Giddings, E. Grushka and P.R. Brown (Editors), Advances in Chromatography, Vol. 27, Marcel Dekker, New York, Basle, 1987, p. 73.
32 R. Däppen, H. Arm and V.R. Meyer, J. Chromatogr., 373 (1986) 1.