Journal of Chromatography, 644 (1993) 315–320
Elsevier Science Publishers B.V., Amsterdam

CHROM. 25 192

Direct column liquid chromatographic enantiomer separation of the coumarin anticoagulants phenprocoumon, warfarin, acenocoumarol and metabolites on an α_1-acid glycoprotein chiral stationary phase

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(First received January 15th, 1993; revised manuscript received April 15th, 1993)

ABSTRACT

The enantiomers of the racemic coumarin anticoagulants phenprocoumon (PH) and metabolites (4′-, 6-, 7- and 8-hydroxy-PH), warfarin (WA) and metabolites (6-, 7-hydroxy-WA and the two diastereomeric WA alcohols) and acenocoumarol were resolved by column liquid chromatography using an immobilized α_1-acid glycoprotein stationary phase; elution was performed using a phosphate buffer and isopropanol gradient with and without dimethyloctylamine as modifier, and detection by ultraviolet or fluorescence. The advantages of this method are: the procedure is simple and fast and does not require pre-column derivatization; the configuration of the enantiomers can be assigned by comparison with a reference sample with already known absolute configuration; the optical purities of these compounds can be analysed with high sensitivity; the method can be applied to the determination of the enantiomers in biological samples.

INTRODUCTION

Column liquid chromatographic (HPLC) separation of enantiomers on chiral stationary phases has been extensively investigated during recent years since many bioactive substances are chiral and enantiomers may show different biological activities [1–4].

The 4-hydroxycoumarin oral anticoagulants phenprocoumon (PH) (**1**), warfarin (WA) (**7**) and acenocoumarol (AC) (**13**) (Fig. 1) are used in human therapy for the treatment and prevention of thromboembolic disorders [5,6]. These drugs are chiral but are available and administered as racemates [7].

Pharmacokinetic studies concerning the enantioselective elimination of coumarin anticoagulants after racemic drug administration require assays with resolution of enantiomers [7–10]. WA and PH have been previously resolved by HPLC using chiral phases [11–13]; however, only the metabolites from WA have been resolved, using a lengthy procedure with pre-column derivatization, separation by normal-phase HPLC and detection by fluorescence after post-column aminolysis [14].

α_1-Acid glycoprotein chiral stationary phases

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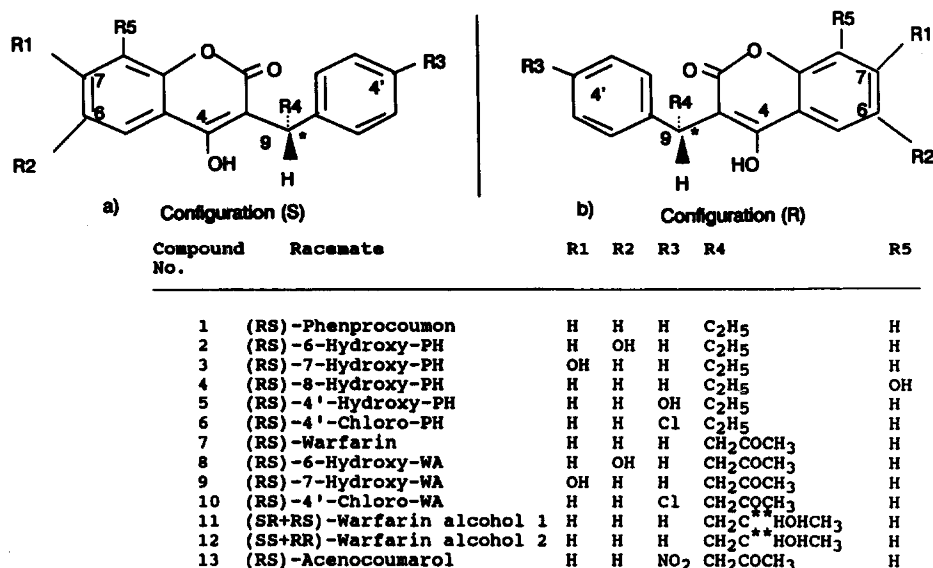

Compound No.	Racemate	R1	R2	R3	R4	R5
1	(RS)-Phenprocoumon	H	H	H	C_2H_5	H
2	(RS)-6-Hydroxy-PH	H	OH	H	C_2H_5	H
3	(RS)-7-Hydroxy-PH	OH	H	H	C_2H_5	H
4	(RS)-8-Hydroxy-PH	H	H	H	C_2H_5	OH
5	(RS)-4'-Hydroxy-PH	H	H	OH	C_2H_5	H
6	(RS)-4'-Chloro-PH	H	H	Cl	C_2H_5	H
7	(RS)-Warfarin	H	H	H	CH_2COCH_3	H
8	(RS)-6-Hydroxy-WA	H	OH	H	CH_2COCH_3	H
9	(RS)-7-Hydroxy-WA	OH	H	H	CH_2COCH_3	H
10	(RS)-4'-Chloro-WA	H	H	Cl	CH_2COCH_3	H
11	(SR+RS)-Warfarin alcohol 1	H	H	H	$CH_2C^{**}HOHCH_3$	H
12	(SS+RR)-Warfarin alcohol 2	H	H	H	$CH_2C^{**}HOHCH_3$	H
13	(RS)-Acenocoumarol	H	H	NO_2	CH_2COCH_3	H

Fig. 1. Structures and configurations of oral anticoagulants and their metabolites [★ and ★★ are chiral centres at C-9 and C-11, respectively; compound numbers followed by a or b correspond to a configuration at C-9 of (*S*) or (*R*), respectively].

have been used for the separation of enantiomers and stereoisomers of a large number of compounds [15–18]; in the present study we report on the use of this phase for the direct HPLC separation of the enantiomers of PH, WA, their metabolites and AC.

EXPERIMENTAL

Chemicals

Racemic warfarin (**7**) and 4'-chlorowarfarin (**10**) were commercially available (Sigma Chemie, Deisenhofen, Germany); (*RS*)-phenprocoumon (**1**), (*S*)-(−)-phenprocoumon (**1a**), (*R*)-(+)-phenprocoumon (**1b**) and (*RS*)-4'-chloro-phenprocoumon (**6**) were gifts from Hoffman-La Roche (Basle, Switzerland); (*S*)-(−)-acenocoumarol (**13a**) and (*S*)-(−)-warfarin (**7a**) were gifts from Dr. H.H.W. Thijssen, (Department of Pharmacology, University of Limburg, Netherlands); racemic acenocoumarol (**13**) was obtained from Ciba-Geigy (Wehr, Germany). Racemic hydroxy-phenprocoumon metabolites (6-OH-PH, 7-OH-PH, 8-OH-PH and 4'-OH-PH) (**2–5**), racemic warfarin metabolites (6-OH-WA, 7-OH-WA) (**8, 9**) and the racemic diastereomeric warfarin alcohols 1 and 2 (**11, 12**) were obtained in our laboratories by chemical synthesis [19–21].

WA alcohols 1 and 2 (**11, 12**) were obtained by reduction of racemic WA (**7**) with sodium borohydride, separation by silica gel column chromatography and recrystallization [21]; alcohol 1 (**11**) (isolated from fractions 11–24) showed m.p. 169–173°C (Lit. [21], 173–175°C), R_F 0.49 (Lit. [21], 0.46), ^{1}H NMR (in C^2HCl_3), δ(ppm) (No. of protons)(multiplicity)(assignment): 7.0–7.8 (9H) (m) (aromatic), 4.60 (1H) (d,d) (C9-H), 3.8 (1H) (m) (C11-H), 2.1–2.5 (2H) (m) (C10-HH), 1.30 (3H) (d) (C12C-H3); alcohol 2 (**12**) (isolated from fractions 32–56) showed m.p. 159–165°C (Lit [21], 162–168°C), R_F 0.39 (TLC, Lit. [21], 0.21), ^{1}H NMR (in C^2HCl_3): 7.2–7.8 (9H) (m) (aromatic), 4.70 (1H) (tr) (C9-H), 4.0 (1H) (m) (C11-H), 2.1–2.6 (2H) (m) (C10-HH), 1.35 (3H) (d) (C12C-H3); UV spectra of **11** and **12** were identical to those reported in the literature [22]. Warfarin alcohols 1a (*SR*) (**11a**) and 2a (*SS*) (**12a**) were obtained similarly by reduction of optically pure (*S*)-(−)-WA (**7a**) and were a gift from Dr. H.H.W. Thijssen.

N,N-Dimethyloctylamine (DMOA) (99%) was obtained from Fluka Feinchemikalien (Neu-Ulm, Germany); reagents and solvents for chro-

matography were analytical grade. Solvents for HPLC were filtered and degassed with helium before chromatography.

Equipment and chromatographic conditions

HPLC analysis was performed with a Model 1090 M liquid chromatograph with ternary solvent delivery system, autosampler, UV–visible photodiode-array detector, fluorescence detector M1046 and a Model 79994A computer workstation for system control and data handling (Hewlett Packard, Waldbronn, Germany). The photodiode-array detection (DAD) wavelengths were set at 276 and 312 nm and on-line spectra were recorded in the range 210–400 nm; fluorescence excitation and emission wavelengths were set at 292 and 380 nm, respectively; the enantiomers were separated with a Chiral-AGP column (100×4 mm I.D., spherical 5-μm particles; ChromTech, Norsborg, Sweden) and a guard column (10×3 mm) filled with the same material.

The following elution systems were used. Elution system 1: solvent A, 0.01 *M* aqueous phosphate buffer pH 7.0; solvent B, 2-propanol. Elution system 2: solvent A, 0.1 *M* aqueous phosphate buffer pH 7.0; solvent B, 2-propanol. Elution system 3: solvent A, 0.01 *M* aqueous phosphate buffer pH 7.0 modified with 0.001 *M* dimethyloctylamine; solvent B, 2-propanol modified with 0.001 *M* dimethyloctylamine. For all elution systems the same linear gradient parameters were used: solvent B 0–20% in 10 min, 15 min with 20% B and 10 min equilibration with 100% solvent A before the next injection. The solvent flow-rate was 0.9 ml/min and the operating pressure and temperature were 10–12 MPa and 20–25°C, respectively. After an analysis series the column was washed with an aqueous 10% 2-propanol solution for 15 min. Aliquots of 1–2 μl of methanolic solutions were injected; larger volumes were injected in phosphate buffer solutions.

Thin-layer chromatography was run on silica gel plates with the solvent toluol–ethyl formate–formic acid (10:5:1; v/v/v) [22] and the spots were visualized under an ultraviolet lamp.

^{1}H NMR spectroscopy was performed on a Bruker Model FT AM-360 instrument at 360 MHz; substances were dissolved in deuterated chloroform or dimethylsulphoxide and tetramethylsilane was used as reference; spectra were obtained by Dr. C. Deus and Professor Dr. H. Friebolin (Institute of Organic Chemistry, University of Heidelberg, Germany); UV spectra (in methanol acidified with 1% 0.1 *M* hydrochloric acid) were run on a Kontron Model Uvikon 910 spectrophotometer.

RESULTS AND DISCUSSION

Enantiomeric separation

The structures of the oral anticoagulants and metabolites are given in Fig. 1. HPLC separation of the enantiomers of the racemic parent drugs PH (**1**), WA (**7**) and AC (**13**) are shown in Fig. 2A–C; Fig. 3 shows the liquid chromatographic separation of some metabolite racemates; Fig. 3A is from 4′-OH-PH (**5**), Fig. 3B from 6-OH-PH (**8**) and Fig. 3C from WA alcohol 2 (**12**). Several solvent combinations and elution systems were evaluated by varying the pH, ionic strength and modifier; elution system 3 showed the best enantiomeric separations of the individual racemates, with the exception of 6-OH-WA (**8**) and 7-OH-WA (**9**), which could, however, be separated with systems 1 and 2, respectively. 4′-Chloro-PH (**6**) and 4′-chloro-WA (**10**) were also analysed as they are used as internal standard for total PH and WA assays; 4′-chloro-WA (**10**) could not be resolved. The (*S*) enantiomers of PH (**1a**) and WA (**7a**) eluted before their respective (*R*) enantiomers, but for AC (**13**) the elution order is reversed (Table I). Chromatographic data are shown in Table I. Only small volumes (1–2 μl) of solutions in organic solvents can be injected due to band broadening of chromatographic peaks; aqueous solutions must be used when injecting larger volumes. Retention times are reproducible within ±2%. The limit of detection of PH, WA and their metabolites using fluorescence detection, and of AC with UV detection, was 10–20 ng; the reported main metabolites from AC, 6-hydroxy-AC and 7-hydroxy-AC, were not available. No racemization was detectable during the solution or chromatographic procedures of pure enantiomers. The standard chromatographic systems and pa-

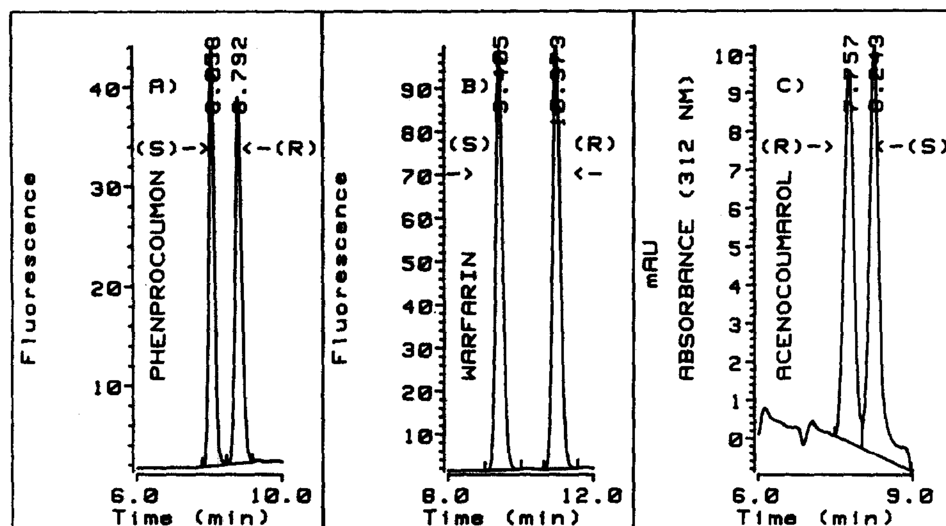

Fig. 2. HPLC separation of the enantiomers of racemic coumarin anticoagulants (elution system 3). (A) Phenprocoumon (1); (B) warfarin (7); (C) acenocoumarol (13). Configuration assignments are indicated in the chromatograms.

rameters mentioned in the Experimental section can, however, be further modified and optimized for a specific separation. Although limitations and drawbacks of protein-derived chiral stationary phases have been reported [2,3], the present chromatographic conditions showed higher selectivity for the direct separation of coumarin anticoagulants and metabolites than the previously used chiral phase of the donor–acceptor type [13]; stationary phase stability is satisfactory

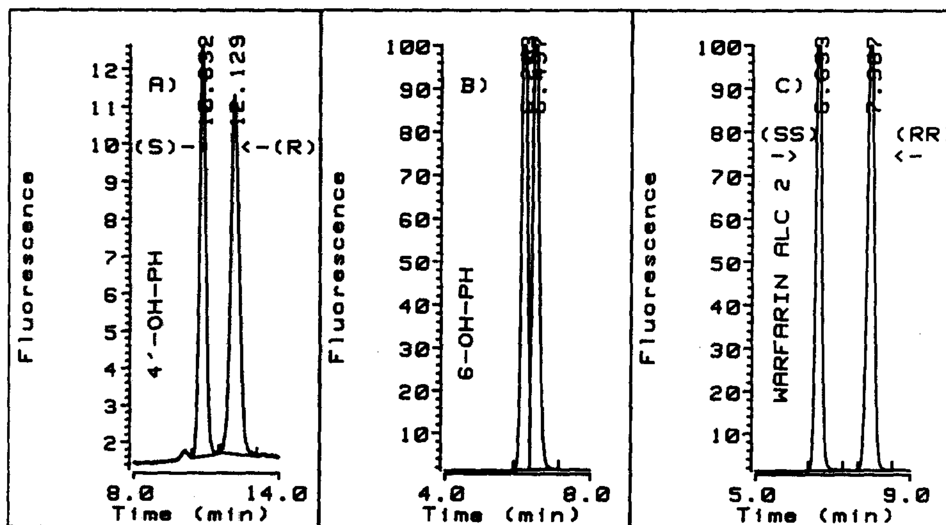

Fig. 3. HPLC separation of the enantiomers of some racemic coumarin anticoagulant metabolites (elution system 3). (A) 4′-Hydroxyphenprocoumon (5); (B) 6-hydroxyphenprocoumon (2); (C) warfarin alcohol 2 (12).

TABLE I

COLUMN CAPACITY FACTORS (k') AND SELECTIVITIES (α) FOR THE ENANTIOMERIC SEPARATION OF COUMARIN ANTICOAGULANTS AND THEIR METABOLITES

N.S. = No separation; (*R*) and (*S*) C-9 configuration assignments.

Compound No.	Elution system 1			Elution system 2			Elution system 3		
	k'_1	k'_2	α	k'_1	k'_2	α	k'_1	k'_2	α
1	8.02 (*S*)	9.23 (*R*)	1.15	9.13 (*S*)	10.70 (*R*)	1.17	7.15 (*S*)	8.51 (*R*)	1.18
2	6.80 (N.S.)			5.65 (N.S.)			4.36	4.63	1.06
3	4.03	4.20	1.04	5.13 (N.S.)			2.82	2.92	1.03
4	4.78	5.09	1.06	5.90	6.03	1.02	4.69	5.08	1.08
5	9.24	10.77	1.16	10.51	13.02	1.23	8.36	9.48	1.13
6	12.7 (N.S.)			10.97 (N.S.)			8.94	9.15	1.02
7	6.92 (*S*)	7.57 (*R*)	1.09	7.67 (*S*)	8.44 (*R*)	1.10	6.04 (*S*)	6.67 (*R*)	1.10
8	3.44	3.70	1.07	3.98 (N.S.)			2.82	2.92	1.03
9	2.94	3.14	1.06	3.33	3.59	1.07	2.49 (N.S.)		
10	11.10 (N.S.)			8.92 (N.S.)			7.35 (N.S.)		
11	6.50 (*SR*)	7.07(*RS*)	1.08	6.78 (*SR*)	7.60(*RS*)	1.12	5.42 (*SR*)	6.00(*RS*)	1.10
12	5.83 (*SS*)	7.07 (*RR*)	1.21	6.15 (*SS*)	7.47 (*RR*)	1.21	4.74 (*SS*)	5.91 (*RR*)	1.24
13	6.67 (*R*)	8.14 (*S*)	1.21	7.77 (*R*)	8.17 (*S*)	1.05	5.72 (*R*)	6.14 (*S*)	1.07

as more than 800 analyses were performed before loss of column efficiency.

Configuration assignment

The absolute configuration of the enantiomers of PH, WA and AC (**1**, **7**, **13**) was determined previously by chemical and chiroptical methods [23–26]. The configurations of the parent drugs were assigned in the present work by injecting the pure enantiomers (**1a**, **1b**, **7a**, **11a**, **12a**, **13a**) in the HPLC system and comparing their retention times and UV on-line spectra; the present HPLC method allows the determination of the configuration of the anticoagulant drugs with small samples.

However, the absolute configuration of the enantiomers of the phenolic metabolites from PH (**2–5**) and WA (**8**,**9**) has not been assigned yet and pure metabolite enantiomers were not available. The racemic diastereomeric warfarin alcohols 1 (**11**) (9*R*, 11*S* + 9*S*, 11*R*) and 2 (**12**) (9*R*, 11*R* + 9*S*, 11*S*) were obtained by chemical reduction of racemic warfarin and column chromatography according to Chan *et al.* [21]; the chromatographic behaviour and the spectral and physical properties of our synthetic compounds corresponded to that of the literature [21,22]; the denominations warfarin alcohols 1 and 2 were based on migration rates on silica and have been retained. The optically pure diastereomeric warfarin alcohols 1a (**11a**) (9*S*, 11*R*) and 2a (**12a**) (9*S*, 11*S*) were obtained by chemical reduction of the (*S*) (−) WA enantiomer.

Optical purity

The optical purities of the compounds can be evaluated quantitatively; a 1% impurity of one of the enantiomers can be detected in the presence of the antipode with less material and higher sensitivity than in polarimetry.

Analysis of biological samples

The HPLC enantiomer separation of the coumarin anticoagulants and their metabolites is fast and simple, and presents advantages over those that require derivatization [10].

GC–MS analysis of methylated WA and PH and their metabolites has been reported but this requires the synthesis and administration of

specific ^{13}C-labelled enantiomer mixtures (pseudoracemates) of the parent drugs [27,28]; however, these assays are not applicable to studies with patients on long-term therapy with racemic anticoagulant administration [7].

The described HPLC method can be applied to biological samples; although enantiomer pairs are well resolved, different metabolites may overlap during chromatography (see Table I).

CONCLUSIONS

Several advantages of the above-described method for the direct liquid chromatographic separation of the coumarin anticoagulant enantiomers of phenprocoumon, warfarin, acenocoumarol and metabolites on α_1-acid glycoprotein chiral stationary phase are reported: it is a simple and fast procedure not requiring pre-column derivatization; the absolute configuration of the parent drugs can be assigned using small amounts of material; the optical purities of these compounds can be determined with high sensitivity; it can be applied to the determination of enantiomers in biological samples.

ACKNOWLEDGEMENTS

We thank Dr. H.H.W. Thijssen (Department of Pharmacology, University of Limburg, Netherlands), Hoffman-La Roche (Basle, Switzerland) and Ciba-Geigy (Wehr, Germany) for samples, Dr. C. Deus and Professor Dr. H. Friebolin (Institute of Organic Chemistry, University of Heidelberg, Germany) for the ^{1}H NMR spectra.

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