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Sensitive stereospecific determination of acenocoumarol and phenprocoumon in plasma by high-performance liquid chromatography

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Abstract

We describe a normal-phase HPLC method for the stereospecific determination of *R*- and *S*-acenocoumarol and *R*- and *S*-phenprocoumon with *S*-warfarin as internal standard. The compounds were separated using a Whelk-O1 chiral stationary phase, detected by UV at 310 nm and quantified in the internal standard mode. Linearity was verified for acenocoumarol in the range of 15–2000 μg/l and for phenprocoumon from 15 to 2200 μg/l, respectively. The detection limits were 5 μg/l for all compounds. The recovery was >84% for *R*- and *S*-acenocoumarol and >74% for *R*- and *S*-phenprocoumon. The imprecision (C.V.) (50–1800 μg/l) for *R*- and *S*-acenocoumarol was <4.7% within-day and <7.8% between-day. For *R*- and *S*-phenprocoumon the respective values were <5.6% and <5.9%. The accuracy for all compounds was 96.5–110%.

Keywords: Enantiomer separation; Acenocoumarol; Phenprocoumon

1. Introduction

Acenocoumarol, phenprocoumon and warfarin are oral anticoagulants which are in widespread use all over the world (Fig. 1). These drugs are used as racemates, with both stereoisomers having different pharmacokinetics and/or pharmacodynamic effects. Because of the very high clearance of *S*-acenocoumarol, the pharmacodynamics in a clinical setting mainly depend on the *R*-isomer. By contrast the pharamcokinetics of both phenprocoumon isomers are comparable, but *S*-phenprocoumon is more potent than the *R*-isomer. *S*-warfarin is also more potent than *R*-warfarin but has a shorter half-life as compared to the *R*-isomer. All these drugs are highly protein bound (>98%) [1].

Usually the monitoring of these drugs in patients is done by their pharmacodynamic effects (prothrombin time). However, there are several situations where monitoring of the drugs itself is necessary. One of these conditions is a pharmacokinetic interaction study. Oral anticoagulants have a narrow

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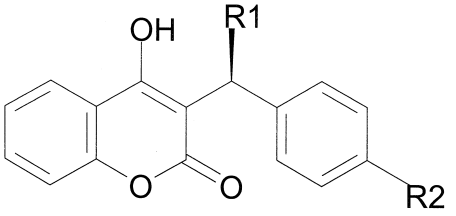

	R1	R2
Acenocoumarol	CH_3COCH_2	NO_2
Phenprocoumon	CH_3CH_2	H
Warfarin	CH_3COCH_2	H

Fig. 1. Chemical structure of acenocoumarol, phenprocoumon and warfarin.

therapeutic index. Pharmacokinetic interactions can occur by drug influences on the anticoagulant's metabolism and/or its binding to plasma albumin [2] resulting in a change of the anticoagulants pharmacokinetics. Other cases where monitoring of anticoagulants has been shown to be useful are patients with an increased prothrombin time which deny the intake of oral anticoagulants. Here only confirmation of presence of an anticoagulant in blood can proof the abuse. Measurement of anticoagulant serum concentrations can also help to differentiate between non-compliance and genuine anticoagulant resistance.

To address these questions, accurate and sensitive methods are needed to determine the stereoisomers of acenocoumarol, phenprocoumon and warfarin in patient blood specimens.

Two stereospecific HPLC methods [3,4] have been developed for the determination of acenocoumarol. These methods suffered from disadvantages as low sensitivity [4] or the need for derivatisation [3]. One HPLC method has been described for the separation of the enantiomers of phenprocoumon and warfarin [5]. With this method baseline chromatographic separation of the enantiomers was not achieved and the sensitivity was accordingly low.

In consideration of two previous publications on the analysis of anticoagulants with GC–MS [6] and the separation of enantiomers with HPLC [7] a new high-performance liquid chromatography (HPLC) method for the simultaneous determination of the enantiomers of acenocoumarol and phenprocoumon was developed using the Whelk-O1 chiral stationary phase. The stereoisomers of warfarin were used as internal standards. Because all these drugs are highly protein bound, the method was also used to determine the free fraction of acenocoumarol and phenprocoumon in plasma water after separation by ultrafiltration [1,8].

2. Experimental

2.1. Drugs and reagents

HPLC grade *n*-hexane, acetone, ethanol and toluene for trace analysis were obtained from Scharlau (Barcelona, Spain); acetic acid was obtained from Fluka (Buchs, Switzerland); sodium hydroxide (p.a.) and hydrochloric acid (p.a.) were obtained from Merck ABS (Dietikon, Switzerland); *R*/*S*-warfarin was obtained from Sigma (Buchs, Switzerland); *R*-acenocoumarol and *S*-acenocoumarol were generous gifts from Novartis (Basel, Switzerland); *R*-phenprocoumon and *S*-phenprocoumon were generous gifts from Roche (Basel, Switzerland).

2.2. Chromatographic conditions

The HPLC system consisted of a 9010 pump, a 9100 autosampler and a 9050 UV–VIS-detector (Varian, Sunnyvale, CA, USA). The detector was set at 310 nm and the autosampler was equipped with a 100 μl loop. The enantiomers of acenocoumarol, phenprocoumon and warfarin (IS) were separated using a S,S-Whelk-01, 5 μm particle size column (25 cm×4 mm, LiChroCART, Merck, Dietikon, Switzerland), protected with a guard column (4×4 mm, LiChrospher 100 DIOL, 5 μm, Merck, Dietikon, Switzerland).

Eluent A consisted of *n*-hexane–ethanol (90:10, v/v) containing 0.5% acetic acid, eluent B of *n*-hexane–ethanol (60:40, v/v) containing 0.5% acetic acid. The mobile phase was linearly mixed in a gradient system starting with 5% eluent B, 95% eluent A changing to 50% eluent B, 50% eluent A during 20 min, during five additional minutes eluent B was increased to 100% and then maintained for 10

min. The flow-rate of the mobile phase was set at 1.0 ml/min. The enantiomeric elution order of the analytes in the chromatogram has been determined by injecting the different enantiomers separately.

2.3. Sample preparation

To 1 ml of plasma (standards, controls or samples) 100 μl of internal standard (1 μg *R*/*S*-warfarin/100 μl 0.05 *M* sodium hydroxide in water) was added and the pH of the mixture was adjusted to 3.5 with 1 *M* hydrochloric acid. Extraction was performed with 5 ml toluene on a horizontal shaker (Infors HAT, Infors, Bottmingen, Switzerland) during 20 min. After centrifugation for 5 min at 1000 *g* the organic layer was separated and dried by evaporation (Rotavapor, Büchi, Flawil, Switzerland) and the residue was dissolved in 150 μl of eluent A.

For quantification standard samples were prepared by adding the appropriate amount of *R*- and *S*-acenocoumarol or *R*- and *S*-phenprocoumon. These standard samples were extracted as described above and the standard curves plotted as the peak area ratio of the respective anticoagulant to the internal standard (*S*-warfarin). To assess linearity, the line of best fit was determined by least square regression. The analysis of all plasma samples has been performed in duplicate on two different days.

The expected free fraction of both anticoagulants was very low because of a protein binding of >98%. Therefore the in-vitro determination of the free fractions had to be performed at concentrations higher than those obtained in-vivo after application of a single dose of acenocoumarol or phenprocoumon [9,10]. Two different amounts of *R*- and *S*-acenocoumarol or *R*- and *S*-phenprocoumon, respectively were added to plasma samples of volunteers obtained before anticoagulant administration.

For the lower anticoagulant concentration either 4.5 μg *R*-acenocoumarol (1500 μg/l) and 4.5 μg *S*-acenocoumarol (1500 μg/l) were added to 3 ml heparin plasma or 4.5 μg *R*-phenprocoumon (1500 μg/l) and 4.5 μg *S*-phenprocoumon (1500 μg/l), respectively. For the higher anticoagulant concentration either 10 μg *R*-acenocoumarol (5000 μg/l) and 10 μg *S*-acenocoumarol (5000 μg/l) were added to 2 ml heparin plasma or 10 μg *R*-phenprocoumon (5000 μg/l) and 10 μg *S*-phenprocoumon (5000 μg/l), respectively. All samples were incubated for 30 min at room temperature.

To determine the total amount of drug in the respective plasma sample, 0.5 ml of spiked plasma was removed from the samples with the lower anticoagulant concentrations and 0.25 ml of spiked plasma from the samples with the higher anticoagulant concentration. To these samples drug-free plasma was added to a total amount of 1 ml. These plasma samples were analysed as described above.

For the determination of the anticoagulant concentration in plasma water (concentration of the free drug) 2.5 ml of the samples with the lower anticoagulant concentration and 1.75 ml of the samples with the higher anticoagulant concentration were ultrafiltrated using Centrifree tubes (cut-off 30 000 Da, Amicon, Beverly, MA, USA) centrifuged for 40 min at 2000 *g* and 25°C [8]. The exact volume of the ultrafiltrate was determined, 50 μl of internal standard (0.1 μg *R*/*S*-warfarin/100 μl 0.05 *M* sodium hydroxide in water) was added and the pH of the mixture adjusted to 3.5 with 1 *M* hydrochloric acid. Extraction was performed twice, each time with 5 ml toluene on a horizontal shaker (Infors HAT, Infors, Bottmingen, Switzerland) for 15 min. After centrifugation for 5 min at 1000 g the organic layer was separated and dried by evaporation (Rotavapor, Büchi, Flawil, Switzerland) and the residue was dissolved in 150 μl of eluent A.

2.4. Assessment of performance characteristics

2.4.1. Linearity

Corresponding known amounts of an *R*- and *S*-acenocoumarol solution in 0.05 *M* sodium hydroxide were added to human plasma for the preparation of six standards of which the concentrations were ranging from 15 to 2000 μg/l in plasma. Corresponding known amounts of an *R*- and *S*- phenprocoumon solution in 0.05 *M* sodium hydroxide were added to human plasma for the preparation of seven standards of which the concentrations were ranging from 15 to 2200 μg/l in plasma. These standard samples were extracted as described above and the standard curves plotted as the peak area ratio of the respective compound to the internal standard versus concentration. To assess linearity, the line of best fit was determined by least square regression.

The standards for the determination of the anticoagulants in plasma water were ranging from 15 to 100 μg/l *R*- and *S*-acenocoumarol and 15 to 60 μg/l *R*- and *S*-phenprocoumon in plasma water.

2.4.2. Precision and accuracy

To determine the analytical precision, three plasma samples were prepared by addition of either 50, 300 and 1800 μg/l *R*-acenocoumarol and *S*-acenocoumarol or *R*-phenprocoumon and *S*-phenprocoumon. On eight different days a calibration curve for each type of analysis was recorded and the three samples were analysed once. For the determination of the within-day precision, the samples were run five times on the same day. To obtain the within-day and between-day coefficients of variation, mean and standard deviations were calculated for each series of analyses.

The accuracy of the method was assessed by expressing the mean of the assayed concentration for the precision samples as a percentage of the weighed-in concentration.

2.4.3. Recovery

For the determination of the recovery 50, 300 or 1800 ng of *R*-, *S*-acenocoumarol or *R*-, *S*-phenprocoumon were added to 1 ml of plasma ('sample') or to 150 μl of mobile phase ('standard'), respectively. The samples were extracted as described above. The standards were injected directly into the chromatographic system. These analyses were performed six times and the average peak area of each compound of the sample was compared to the corresponding peak area of the standard.

2.4.4. Detection limit

The detection limit of the method was calculated using a signal-to-noise ratio of two. For this purpose, the noise signal was obtained as the amplitude of the peaks from a segment of the chromatogram that preceded each acenocoumarol or phenprocoumon peak, respectively.

2.4.5. Lower limit of quantification

The lower limit of quantification (LLOQ) usually is the concentration of the lowest calibration standard (15 μg/l for all analytes) [11]. The accuracy and precision of the lowest calibration standard was determined by analyzing a sample with 15 ng *R*- and *S*-acenocoumarol and 15 ng *R*- and *S*-phenprocoumon added to 1 ml of plasma. On eight different days a calibration curve for each compound was recorded and the sample analyzed once. The within-day coefficient of variation was calculated and used as a measure of the analytical precision. The accuracy of the LLOQ was determined as the mean of the assayed concentration expressed as a percentage of the weighed-in concentration.

2.5. Interferences

Several commonly co-administered drugs were added to blank plasma in concentrations at the upper therapeutic range. These spiked plasma samples were extracted and analysed as described above. The appearance of a peak at the retention time of one of the enantiomers of the oral anticoagulants was the parameter to decide that there was an interference of the corresponding drug.

2.6. Optical rotation

The optical rotation of all compounds has been determined at the organic chemical institute of the University Zurich using a Perkin-Elmer polarimeter 241 (Perkin-Elmer, Switzerland) with a sodium lamp at the wavelength of 589 nm. The calibration substance was thymidine at a concentration of 10.1 mg/ml water.

3. Results

3.1. Chromatographic separation

Representative chromatograms for *R*- acenocoumarol, *S*- acenocoumarol, *R*-phenprocoumon, *S*-phenprocoumon, *R*-warfarin and *S*-warfarin in are shown in Fig. 2 demonstrating the absence of interfering endogenous substances and baseline separation of all compounds. All peaks were symmetrical and well resolved.

The retention times were: 9.5 min for *S*-phenprocoumon, 11.5 min for *R*-phenprocoumon, 13.5 min for *R*-warfarin, 17 min for *S*-warfarin, 24 min

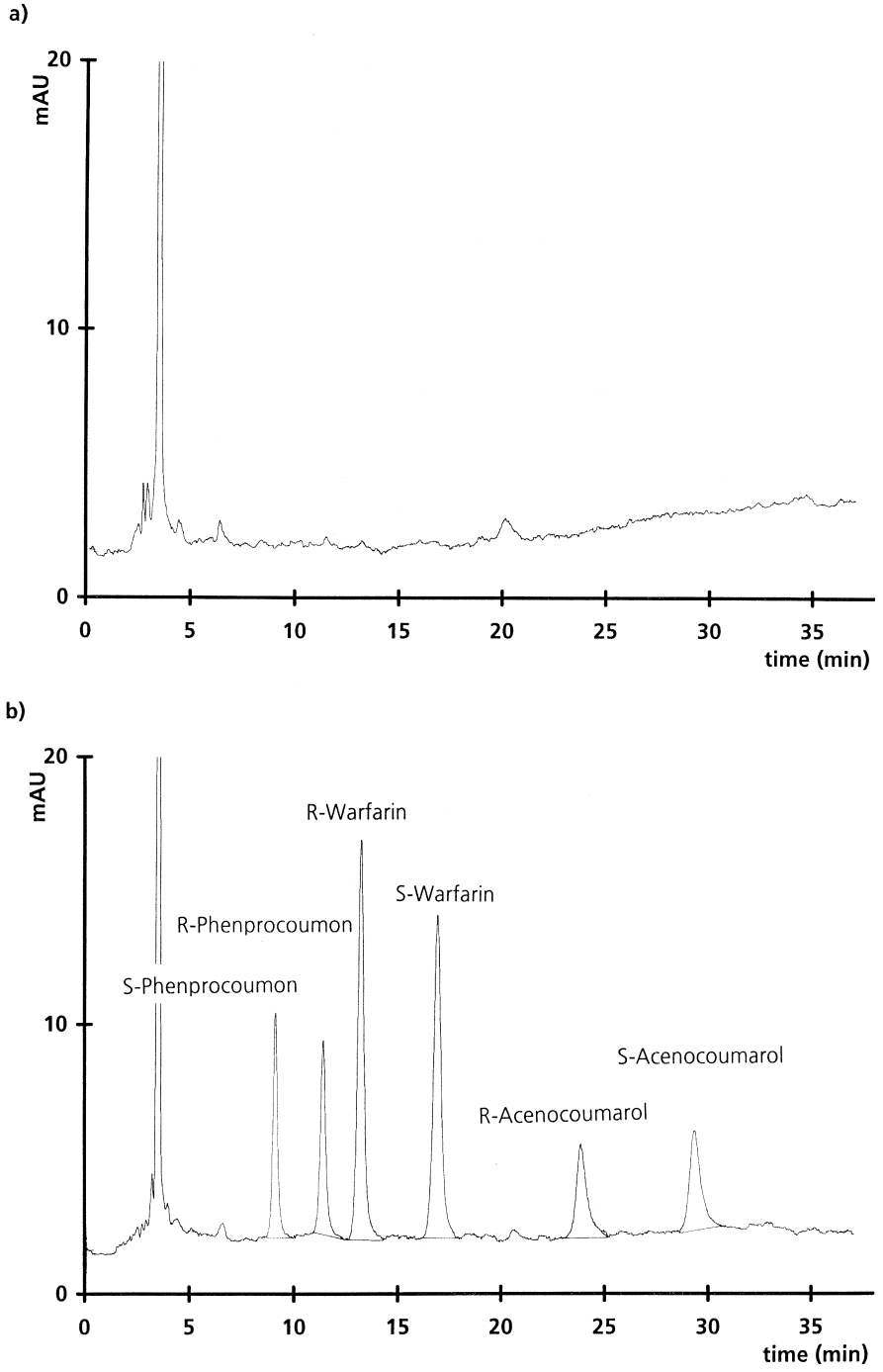

Fig. 2. (a) Chromatogram of a blank plasma sample; (b) Chromatogram of a spiked plasma sample with 250 μg/l *R*- and *S*-acenocoumarol, *R*- and *S*-phenprocoumon and 500 μg/l *R*- and *S*-warfarin; (c) Chromatogram of a healthy volunteer's plasma sample containing 39 μg/l *R*-acenocoumarol, 32 μg/l *S*-acenocoumarol and 1 mg/l R/*S*-warfarin as internal standard; (d) Chromatogram of a healthy volunteer's plasma sample containing 38 μg/l *R*-phenprocoumon and 35 μg/l *S*-phenprocoumon and 1 mg/l R/*S*-warfarin as internal standard.

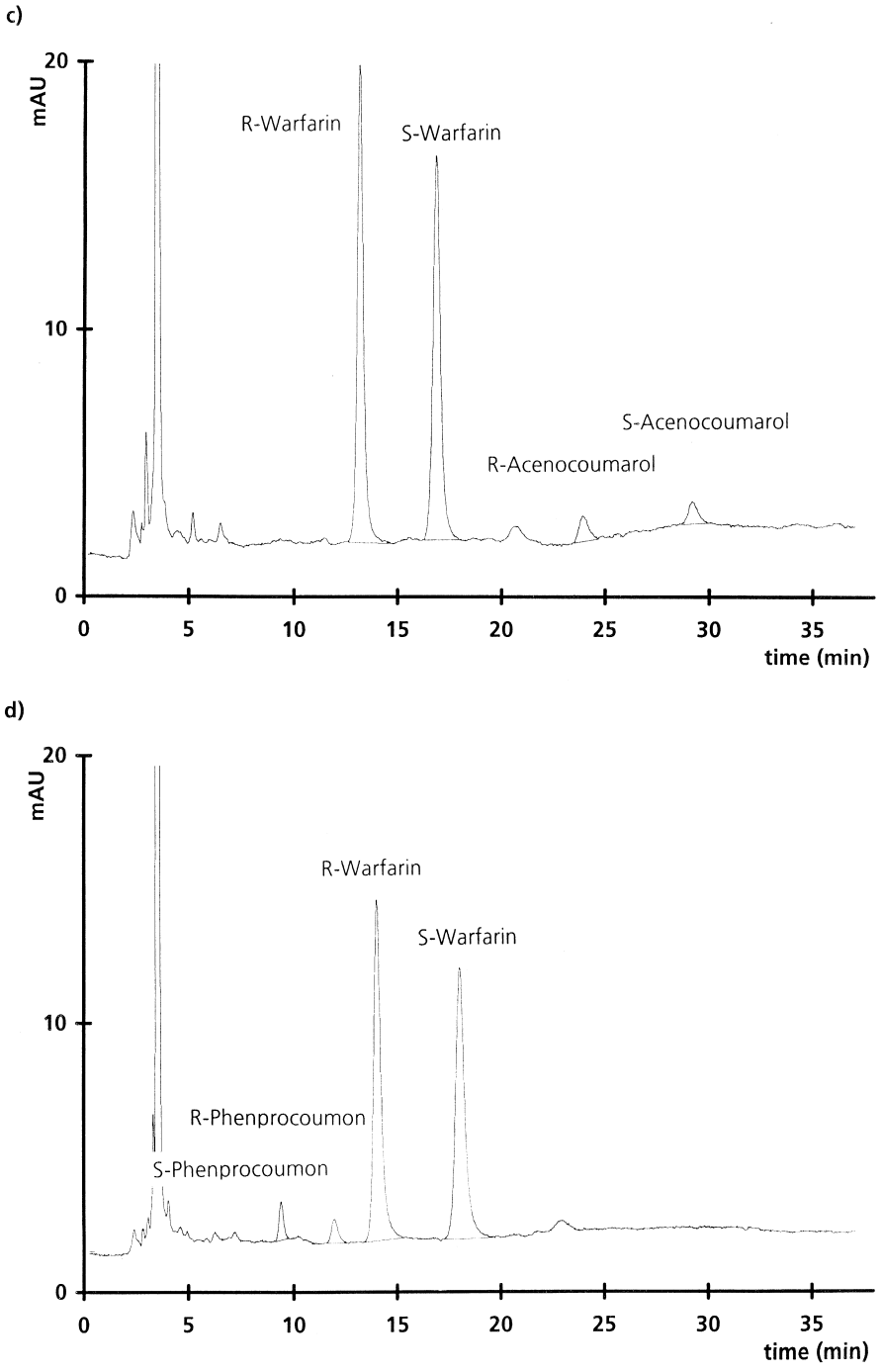

Fig. 2. (*continued*)

for *R*-acenocoumarol and 29 min for *S*-acenocoumarol.

3.2. Performance characteristics

3.2.1. Linearity

The standard curves for *R*-, *S*-acenocoumarol and *R*-, *S*-phenprocoumon in plasma were linear in the range of 15–2000 μg/l and 15–2200 g/l, respectively. Least square regression data of the calibration cures are summarized in Table 1. Residual plots of the different calibration curves are depicted in Fig. 3.

3.2.2. Precision and accuracy

The results of the precision and accuracy experiments are summarised in Table 2. The validation data of all analytes prove that the extraction procedures and the HPLC method were precise and accurate in the range of 50–1800 μg/l. All between-day and within-day coefficients of variation were below 8.0%.

3.2.3. Recovery

The recoveries of *R*-, *S*-acenocoumarol and *R*-, *S*-phenprocoumon are shown in Table 3. *R*- and *S*-acenocoumarol have recoveries >84%, whereas the corresponding values for *R*- and *S*-phenprocoumon are >74%. The recoveries of the ultrafiltrates were 65–70% for all compounds.

3.2.4. Detection limit

The detection limit (signal-to-noise ratio, 2) for plasma samples of 1 ml was 5 μg/l for *R*- and *S*-acenocoumarol and 5 μg/l for *R*- and *S*-phenprocoumon.

3.2.5. Lower limit of quantification

The LLOQ was 15 μg/l for *R*- and *S*-acenocoumarol and *R*-and *S*-phenprocoumon. The coefficients of variation were ranging from 6.6 to 12.2% and the accuracy from 104 to 117%, respectively.

3.3. Application of the method

The applicability of the method was proven by analysing acenocoumarol and phenprocoumon concentrations in an interaction study with lornoxicam [9,10]. The concentration time profile of *R*- and *S*-acenocoumarol or *R*- and *S*-phenprocoumon in two healthy volunteers, after a single dose of 10 mg acenocoumarol or 15 mg phenprocoumon, is shown in Fig. 4a and b, respectively.

3.4. Interferences

The drugs tested for interferences are shown in Table 4. The only interference observed was coelution of carbamazepine with *S*-warfarin.

4. Discussion

The purpose of this study was to develop a method for the sensitive stereospecific determination of the most commonly used oral anticoagulants. In contrast to previously published HPLC protocols, these requirements were successfully met with the method presented here. Furthermore, the assay protocol is particularly convenient because of extremely simple sample preparation and no requirement for derivatisation or special equipment. The performance characteristics show that the assay is accurate and repeatable. The shelf-life of the column was extremely long, according to our experience more than 2000 injections of plasma samples could be performed with the same column. In order to obtain a short time of analysis as well as properly separated

Table 1
Least square regression data of *R*-, *S*-acenocoumarol and *R*-, *S*-phenprocoumon

Drug	Slope (mean±SD)	Intercept (mean±SD)	Correlation coefficient (mean±SD)
R-Acenocoumarol	0.0027±0.0001	−0.0195±0.0023	0.9998±0.0001
S-Acenocoumarol	0.0029±0.0001	−0.0293±0.0008	0.9999±0.0001
R-Phenprocoumon	0.0029±0.0001	−0.0046±0.0017	0.9998±0.0001
S-Phenprocoumon	0.0029±0.0001	−0.0048±0.0010	0.9999±0.0001

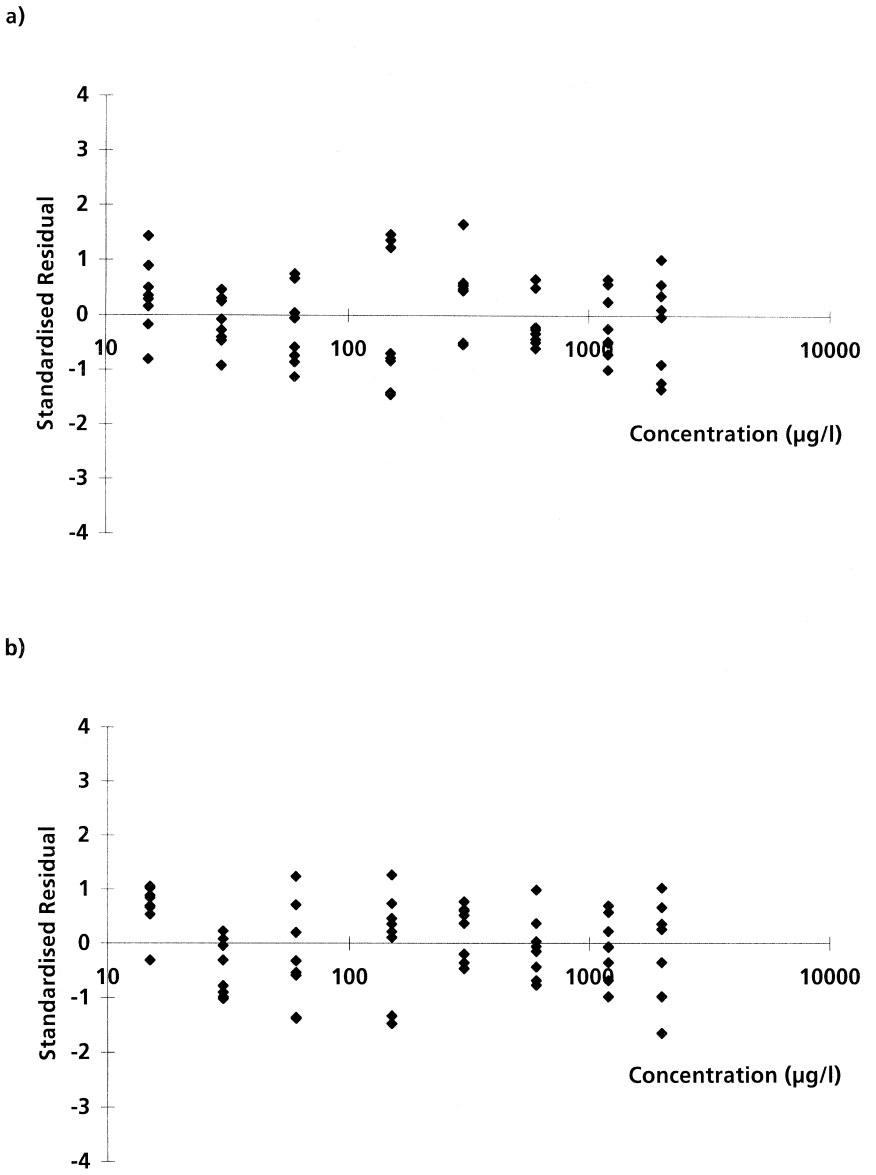

Fig. 3. Residual plots of 8 different calibration curves for (a) *R*-acenocoumarol (b) *S*-acenocoumarol (c) *R*-phenprocoumon (d) *S*-phenprocoumon.

peaks of the different enantiomers, a gradient system of the mobile phase was chosen.

The separation of the stereoisomers of the three commonly used oral anticoagulants is performed on the Whelk-O1 chiral stationary phase. The chiral selector can be viewed as a semirigid framework holding a π-acidic 3,5-dinitrobenzamide group perpendicular to a π-basic polynuclear aromatic group. The amide N–H serves as a hydrogen bond donor and is situated in the cleft formed by the two

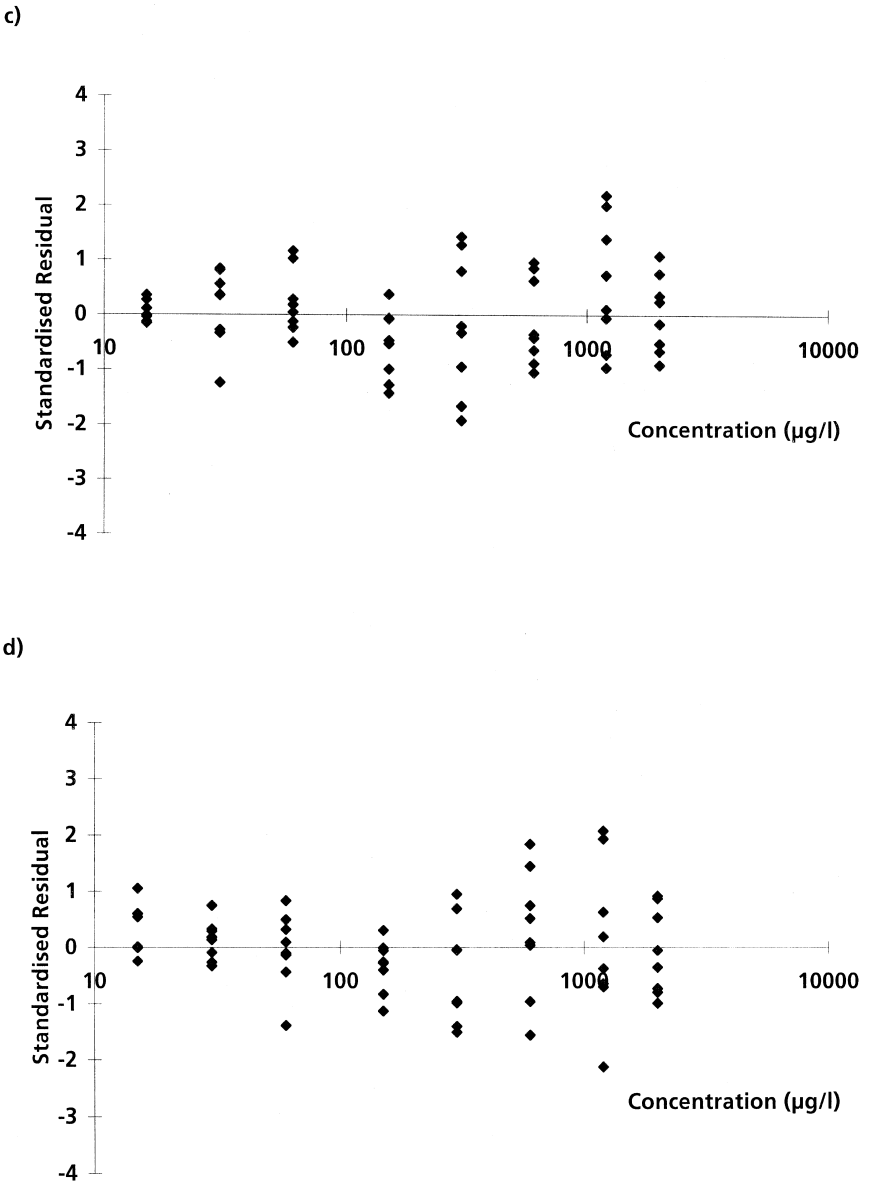

Fig. 3. (*continued*)

aromatic systems. These are capable of simultaneous face to face and face to edge $\pi-\pi$ interaction with an aromatic group present in the analyte [12]. The sequence of the elution of the different stereoisomers of acenocoumarol, phenprocoumon and warfarin can only be partially explained. Most probably the missing keto group in the phenprocoumon molecule is responsible for the different stereospecific elution sequence of this drug.

The different anticoagulants were chemically and

Table 2
Precision and accuracy data of *R*-, *S*-acenocoumarol and *R*-, *S*-phenprocoumon

Drug	Concentration (μg/l)	*n* (μg/l)	Mean	SD (μg/l)	C.V. (%)	Accuracy (%)
R-Acenocoumarol						
Within-day:	50	5	55.0	2.55	4.6	110
	300	5	304	9.76	3.2	101
	1800	5	1779	18.4	1.0	98.8
Between-day:	50	8	50.9	3.95	7.8	102
	300	8	295	5.77	2.0	98.3
	1800	8	1823	41.6	2.3	101
S-Acenocoumarol						
Within-day:	50	5	48.3	2.28	4.7	96.5
	300	5	308	6.32	2.1	103
	1800	5	1799	18.4	1.0	99.9
Between-day:	50	8	52.3	3.19	6.1	105
	300	8	300	12.0	4.0	100
	1800	8	1811	56.0	3.1	101
R-Phenprocoumon						
Within-day:	50	5	54.2	3.06	5.6	108
	300	5	308	6.05	2.0	103
	1800	5	1833	26.3	1.4	102
Between-day:	50	8	56.5	3.35	5.9	113
	300	8	305	5.05	1.7	102
	1800	8	1818	30.5	1.7	101
S-Phenprocoumon						
Within-day:	50	5	51.6	1.50	2.9	103
	300	5	302	11.8	3.9	101
	1800	5	1827	40.3	2.2	102
Between-day:	50	8	52.9	2.47	4.7	106
	300	8	300	6.21	2.1	100
	1800	8	1797	27.7	1.5	99.8

chirally stable. This was confirmed by repeated injections of a freshly thawed standard solution in eluent A in the course of a clinical study lasting four months. During the time of a single run there was no degradation, which has been shown by injecting the calibration samples at the beginning and at the end of the run.

In the presented analyses, heparinized plasma was used as sample material. In a pilot study (data not shown) the concentrations of the anticoagulants in serum and heparinized plasma were compared. The concentrations and recoveries of acenocoumarol, phenprocoumon and warfarin were identical in these two materials.

In order to increase the recovery of acenocoumarol, phenprocoumon and warfarin the use of solid-phase extraction (SPE) using Bond Elut, C_{18} cartridges (Varian) was also evaluated. After prewashing the cartridges with 4 ml distilled water, 4 ml methanol and 4 ml 2% acetic acid, 1 ml plasma containing 100 μl of internal standard (1 μg *R*/*S*-warfarin/100 μl 0.05 *M* sodium hydroxide in water) was applied to the cartridge, followed by 4 ml 1% acetic acid and 4 ml methanol–water (2:8, v/v).

Table 3
Recoveries of *R*-, *S*-acenocoumarol and *R*-, *S*-phenprocoumon ($n=6$)

Drug	50 μg/l	300 μg/l	1800 μg/l
R-Acenocoumarol			
mean	87.1%	92.9%	86.3%
SD	7.5%	2.5%	2.5%
S-Acenocoumarol			
mean	84.3%	96.9%	89.0%
SD	7.8%	2.8%	2.8%
R-Phenprocoumon			
mean	84.6%	83.5%	79.7%
SD	2.9%	2.4%	2.3%
S-Phenprocoumon			
mean	74.4%	82.7%	77.6%
SD	3.8%	2.7%	3.4%

Acenocoumarol, phenprocoumon and warfarin were eluted with 1 ml acetone. The acetone was dried by evaporation (Rotavapor, Büchi, Flawil, Switzerland) and the residue dissolved in 150 μl of eluent A. The recoveries of the SPE extraction were comparable with the results of the single liquid–liquid extraction. In addition, the recovery experiments were repeated with double extraction (4 ml toluene twice), resulting in similar recoveries as compared to the single extraction. Because of the minor expenses liquid–liquid extraction of the plasma samples was used in the study presented.

The recoveries of the ultrafiltrates are smaller than the recoveries of the plasma samples. The binding of the anticoagulants to the ultrafiltration system can be excluded, because there was no decrease of the anticoagulant's concentrations after ultrafiltration of aqueous solutions of the drugs (20 ng of each *R*- and *S*-acenocoumarol or *R*- and *S*-phenprocoumon).

The interferences of different drugs were tested by analysing the parent drugs. In-vivo there exist many metabolites which also could interfere with the presented method. Due to the limited availability of the metabolites it was not possible to test for them.

The method presented here was used and validated

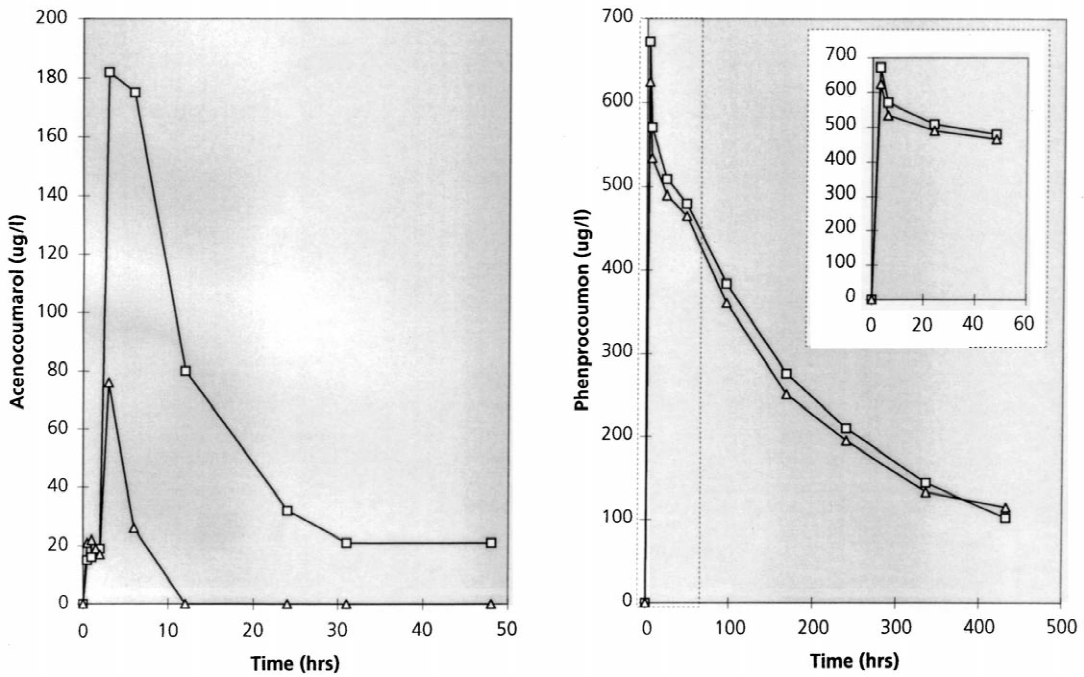

Fig. 4. Concentration versus time curves of *R*- and *S*-acenocoumarol (Fig. 3a) and *R*- and *S*-phenprocoumon (Fig. 3b). □ represents the *R*-stereoisomer, Δ the *S*-stereoisomer.

Table 4
Drugs tested for interferences

Alprazolam	Diclofenac	Levomepromazine	Opipramol	Thiopental
Amitriptyline	Diphenhydramine	Lidocaine	Oxcarbazepine	Thioridazine
Bupivacaine	Doxepin	Lorazepam	Penfluridol	Tolfenamic acid
Carbamazepine	Fentanyl	Maprotiline	Phenobarbital	Topiramate
Chlordiazepoxide	Flunitrazepam	Mefenamic acid	Pipamperone	Trazodone
Citalopram	Flupentixol	Mepivacaine	Promazine	Triazolam
Clobazam	Fluphenazine	Mianserin	Quinine	Trimethoprim
Clomipramine	Flurazepam	Midazolam	Ranitidine	Trimipramine
Clonazepam	Fluvoxamine	Nefazodone	Sertraline	Venlafaxine
Clozapine	Haloperidol	Nordazepam	*S*-Ibuprofen	Zolpidem
Desipramine	Imipramine	Nortriptyline	Sotalol	Zuclopenthixol
Diazepam	Lamotrigine	Olanzapine	Temazepam	

for the analysis of acenocoumarol and phenprocoumon. But it can also be applied for the stereoselective determination of warfarin using one of the other compounds as internal standard.

The results demonstrate that the presented method is a reliable and convenient procedure for the stereoselective quantification of plasma and plasma water concentrations of acenocoumarol, phenprocoumon and warfarin.

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