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Development and validation of a high-performance liquid chromatographic method for the quantitation of warfarin enantiomers in human plasma*

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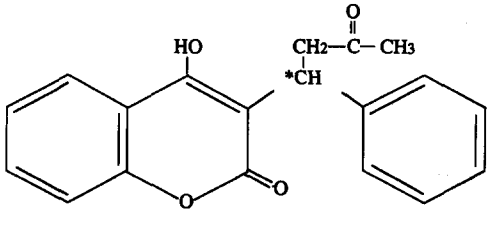
Abstract: An HPLC method for the quantitation of warfarin enantiomers in human plasma has been developed and validated. Baseline separation of *S*- and *R*-warfarin was achieved on a silica-bonded β-cyclodextrin column with a mobile phase of acetonitrile–acetic acid–triethylamine (1000:3:2.5, v/v/v). The detection was performed at 320 nm. The established linearity range was 12.5–2500 ng ml^{-1} ($r > 0.99$). The limit of quantitation was 12.5 ng ml^{-1} for each enantiomer. Inter-day precision and accuracy of 12.5 ng ml^{-1} standards were 12.1% relative standard deviation (RSD) and +0.67% bias for *S*-warfarin and 9.7% RSD and +10.8% bias for *R*-warfarin. The low quality control samples at 37.5 ng ml^{-1} showed 6.9% RSD and 0.0% bias for *S*-warfarin, 7.2% RSD and +0.5% bias for *R*-warfarin. *S*-Naproxen was used as internal standard. Potential metabolites of warfarin were well resolved from *S*- and *R*-warfarin, the internal standard (*S*-naproxen) and each other. The run time was 25 min. The silica-bonded β-cyclodextrin column showed excellent stability; over 1000 samples were injected without significant loss of performance. The column variability test showed that the method can be applied on several batches of β-cyclodextrin columns but not all the β-cyclodextrin columns were suitable for this method.

Keywords: R- *and* S-*warfarin; enantiomers; HPLC chiral separation;* β-*cyclodextrin CSP; column variability.*

Introduction

Warfarin is a coumarin anticoagulant most frequently prescribed for the treatment of thromboembolic disease. It has one asymmetric centre (Fig. 1). The enantiomers differ in their affinity with biological macromolecules resulting in variable degrees of stereoselectivity in transport, anticoagulant potency, metabolism, elimination rate and interaction with other drugs [1, 2].

Warfarin is administered as a racemic mixture. In man, *S*-warfarin was reported to have 2–5 times the anticoagulant potency of *R*-warfarin. The *S*-enantiomer was found to be metabolized faster than *R*-warfarin by the cytochrome P-450 enzymes to 6-, 7- and 8-hydroxywarfarin [3]. 6*R*- and 7*S*-hydroxywarfarin are the major metabolites of warfarin *in vivo* [1]. The stereoselective interaction between warfarin and the cytochrome P-450 system has prompted the interest in many studies of drug interaction and studies using warfarin as a stereochemical probe [4]. The measurements of the warfarin enantiomers in body fluids are necessary for these studies and for chiral pharmacokinetics for a better understanding of the drug concentration and anticoagulant effect relationship.



*Asymmetric Carbon

Figure 1
Structure of warfarin.

Determination of warfarin enantiomers by HPLC methods has been reported. Pre-column derivatizations on the 4-hydroxy group to form diastereomers allowed subsequent separations by HPLC using achiral columns [5–7]. These derivatization methods are time-consuming and may increase assay variability. Chiral derivatization reagents of high quality are costly and some of these are not readily

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available. The differential binding of warfarin enantiomers to chiral macromolecules has been exploited in chiral stationary phase (CSP) separations of *S*- and *R*-warfarin [8–12]. However, direct separation of warfarin enantiomers on protein-based CSP columns suffered from poor sensitivity and short column life [10]. Synthetic CSP columns were used for the separation of warfarin enantiomers in non-biological matrices [13, 14]. The run time was lengthy with broad peaks for the compounds of interest.

We developed a sensitive and reliable HPLC method for the quantitation of warfarin enantiomers in human plasma. Enantioselective separation of *S*- and *R*-warfarin was achieved on a β-cyclodextrin stationary phase with a polar solvent mobile phase. The sample preparation involved a simple organic solvent extraction step. Peak resolution, column stability and reproducibility were excellent. The method has been validated and shown to be rugged for the application to pharmacokinetic and drug interaction studies for warfarin enantiomers.

Experimental

Materials and reagents

Racemic warfarin was from USP, the internal standard (I.S.) *S*-naproxen was from Sigma (St Louis, MO, USA). *S*- and *R*-warfarin, 6- and 7-hydroxywarfarin were kindly donated by the Upjohn Company (Kalamazoo, MI, USA). All organic solvents used were of analytical grade and purchased from Sigma or Fisher (Fair Lawn, NJ, USA). Deionized water was purified by a Nanopure® system from Barnstead. Control EDTA human plasma was purchased from Worldwide Biological (Cincinnati, OH, USA).

Two primary stock solutions of warfarin were prepared from separate weighings for standards and quality control samples (QCs). Methanolic solutions of warfarin primary stock and substocks were prepared under yellow light and stored in aluminum foil-wrapped containers. Working standards were prepared fresh daily by spiking 100 μl 10-fold concentrated solutions into 1.0 ml of blank control EDTA plasma. The final concentrations of *S*- or *R*-warfarin in plasma standards were 12.5, 25, 50, 100, 250, 500, 1000 and 2500 ng ml^{-1}. Three levels of QCs, 37.5, 150 and 1500 ng ml^{-1}, were prepared, aliquoted and stored frozen with the clinical samples to be analysed. Extra sets of QCs were stored in a freezer set at −20°C for long-term storage stability tests.

Instrumentation

The HPLC system consisted of a Beckman 110B solvent delivery module, a Perkin–Elmer ISS-100 autosampler, a Waters 481 LC–UV detector set at 320 nm, and VG® Multichrom data system for VAX®/VMS. The flow rate was 1.0 ml min^{-1}, and the run time was set at 25 min. The analytical column, a β-cyclodextrin column of 25 cm × 0.46 cm i.d. was from Astec (Whippany, NJ, USA). The guard column was a β-cyclodextrin column of 5 cm × 0.46 cm i.d. from Astec. The mobile phase was acetonitrile–glacial acetic acid (HAc)–triethylamine (TEA) (1000:3:2.5, v/v/v).

Data treatment

Chromatograms were measured using a VG® Multichrom data system. The raw data output was acquired on a VG® Chromserver and then transferred to the VAX®/VMS. A weighted $1/y$ linear regression was used to determine slopes, intercepts and correlation coefficients, where y = the ratio of the compound peak height to the internal standard peak height. The resulting parameters were used to calculate concentrations:

$$\text{conc.} = [\text{ratio} - (y\text{-intercept})]/\text{slope}.$$

Extraction procedures

To 1.0 ml plasma sample, 50 μl of I.S. solution (80 μg ml^{-1}) and 700 μl of 1 N sulphuric acid were added. After mixing, 5 ml ethyl ether was added to extract the compounds. The organic extract was evaporated to dryness under nitrogen and reconstituted in 200 μl of acetonitrile. A 50 μl volume was injected onto the HPLC.

Results and Discussion

Resolution

One specific aim of this study was to develop a chiral separation for warfarin with resolution and selectivity with respect to its major metabolites. Figure 2 shows a chromatogram for unextracted racemic warfarin with its major metabolites, 6-hydroxywarfarin and 7-hydroxywarfarin, and I.S. The peaks of *S*- and *R*-warfarin, metabolites, and I.S. were well-resolved. We did not attempt to determine the

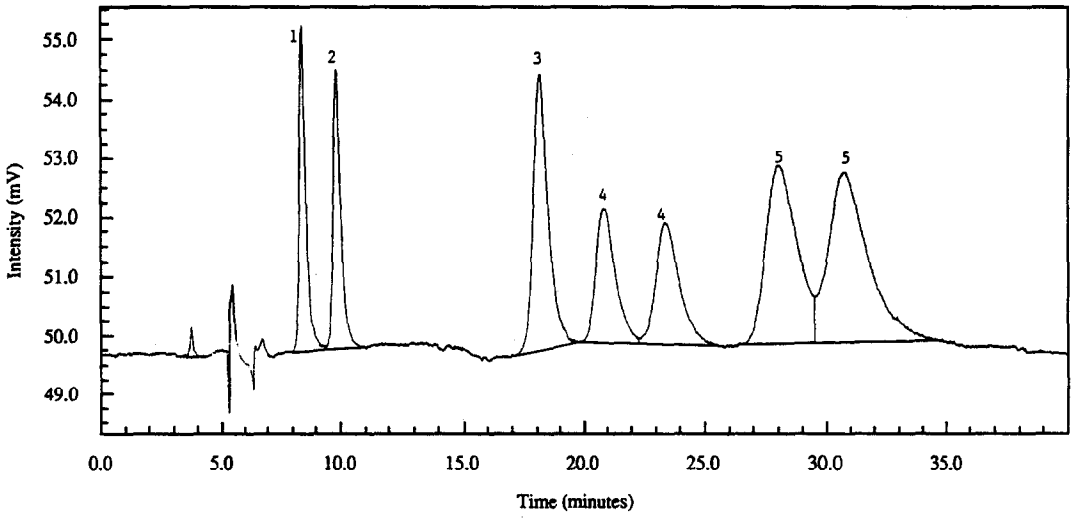

Figure 2
Unextracted racemic warfarin with I.S. and metabolites. Peak identification: 1, *S*-warfarin; 2, *R*-warfarin; 3, I.S.; 4, 6-hydroxywarfarin; 5, 7-hydroxywarfarin. See Experimental for chromatographic conditions.

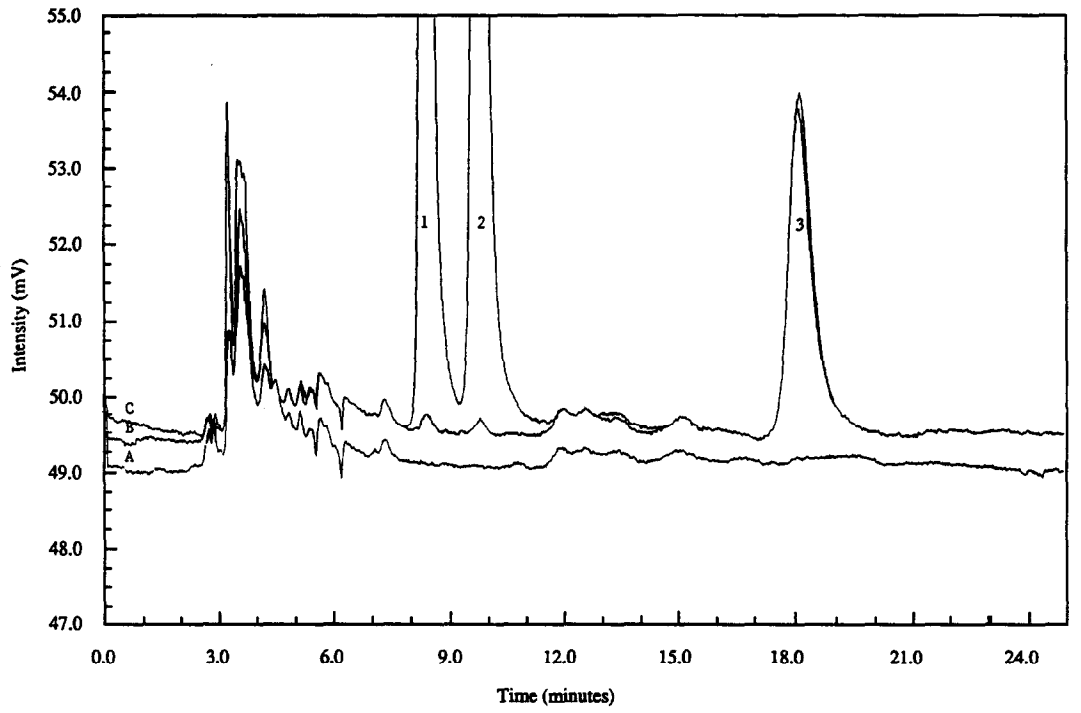

Figure 3
Extracted blank control plasma, standard and QC peak identification. 1, *S*-warfarin; 2, *R*-warfarin; 3, I.S. A, Blank control plasma; B, standard at 12.5 ng ml^{-1} for each enantiomer; C, QC at 1500 ng ml^{-1} for each enantiomer.

elution order for enantiomers of the metabolites since quantitation of the metabolites was not our objective here.

The peaks at *S*- and *R*-warfarin were baseline-resolved, enabling the accurate quantitation of one enantiomer at low concentration in the presence of the other enantiomer at a disproportionately higher concentration. Baseline-resolution of *S*- and *R*-warfarin was not achieved in literature methods [8–14]. Both the HAc and TEA in the mobbile phase are necessary for the excellent resolution of the warfarin enantiomers. Slight changes in the HAc/TEA ratio did not affect the separation.

Figure 3 shows chromatograms of extracted blank control plasma, standard, and quality control. No interference was observed in all eight different lots of control EDTA plasma tested. The chromatograms of the different lots of extracted plasma samples had very little baseline noise compared to the signals of the warfarin peaks. The polar solvent elution used here had the advantage of eluting most of the interfering peaks that were seen in many of the literature methods.

The typical retention times were 8.4, 9.8 and 18.2 min for *S*-warfarin, *R*-warfarin and I.S., respectively. The run time was 25 min. The later eluted 7-hydroxywarfarin peaks did not show interference in the subsequent chromatogram of the next injection. Resolution was achieved in a reasonably short chromatographic time. The system setup was simple, with no need of column-switching and additional pumps.

Extraction recovery

We used a simple single step of liquid–liquid extraction for sample clean-up. Table 1 shows that the overall extraction recoveries were 78.5, 79.4 and 88.5% for *S*-warfarin, *R*-warfarin and the I.S., respectively. This simple extraction step allowed a large number of samples to be processed in a short time. Coupled with the 25-min chromatgraphic run time, 50 samples can be extracted by one analyst and analysed within 24 h.

Validation performance

Six validation curves were run on six separate days over a 3 week period. Table 2 shows the standard curve parameters of slope, *y*- and *x*-intercepts, and correlation coefficients of these curve runs. The performance characteristics are very similar for both *S*- and *R*-warfarin. We observed consistent slopes and excellent correlation coefficients throughout these validation runs. The standards show a linear range of 12.5–2500 ng ml^{-1}, with a limit of quantitation at 12.5 ng ml^{-1} (RSD% of 12.1 for *S*-warfarin and 9.7 for *R*-warfarin). Table 3 shows the accuracy and precision data at each individual standard concentration. Table 4 presents the inter-day and intra-day accuracy and precision of QCs.

The accuracy and precision data show that this method is consistent and reliable with low values in relative error and RSDs for standards and QCs at the entire concentration range. The current standard curve range is suitable in most pharmacokinetic studies.

Stability

Stabilities of processing (freeze–thaw,

Table 1
Recovery of *S*- and *R*-warfarin and internal standard in human plasma

	S-Warfarin (ng ml^{-1})			*R*-Warfarin (ng ml^{-1})			
	12.5	250	2500	12.5	250	2500	Internal standard
Extracted peak height	242	3947	43633	208	3420	37661	4344
RSD (%)	9.4	1.7	3.6	25.3	2.5	3.5	6.8
Unextracted peak height	288	5381	55770	241	4631	48285	4911
RSD (%)	5.9	0.9	3.8	12.1	1.0	3.8	4.9
n	3	3	3	3	3	3	9
% Recovery	84.0	73.4	78.2	86.3	78.9	78.0	88.5
Mean % recovery		78.5			79.4		

Table 2
Inter-day standard curve statistics for *S*- and *R*-warfarin

	S-Warfarin				*R*-Warfarin			
Std curve no.	Slope	*y*-intercept	*x*-intercept	Correl. coeff.	Slope	*y*-intercept	*x*-intercept	Correl. coeff.
A	0.0038	0.0061	−1.5808	0.9939	0.0033	0.0009	−0.2768	0.9939
B	0.0039	−0.0044	1.125	0.9999	0.0034	0.0007	−0.2159	0.9999
C	0.0039	0.0053	−1.3444	0.9995	0.0034	0.0079	−2.3339	0.9997
D	0.0041	0.0127	−3.0916	0.9997	0.0035	−0.0038	1.077	0.9996
E	0.0039	−0.0033	0.8548	0.9996	0.0033	−0.0046	1.383	0.9996
F	0.0039	−0.0006	0.1698	0.9998	0.0034	0.0018	−0.5304	0.9997
Mean	0.0039	0.0026	−0.6445	0.9987	0.0033	0.0004	−0.1495	0.9987
RSD (%)	2.5			0.24	2.2			0.24

Table 3
Inter-day precision and accuracy of *S*- and *R*-warfarin standards

	Standard conc. (ng ml^{-1})							
	12.5	25.0	50.0	100	250	500	1000	2500
S-Warfarin								
Mean	12.6	26.9	48.7	99.8	239	495	1040	2490
RSD (%)	12.1	10.4	3.6	3.5	3.9	1.6	8.3	3.2
RE (%)	+0.67	+7.7	−2.6	−0.20	−4.6	−1.0	+3.7	−0.53
n	6	6	6	6	6	6	6	6
R-Warfarin								
Mean	13.8	25.5	47.2	98.2	240	495	1040	2490
RSD (%)	9.7	5.4	2.6	2.0	3.8	1.7	8.3	3.3
RE (%)	+10.8	−2.1	−5.6	−1.8	−4.0	−1.1	+3.9	−0.40
n	6	6	6	6	6	6	6	6

Table 4
Precision and accuracy of *S*- and *R*-warfarin quality controls

	S-Warfarin (ng ml^{-1})			*R*-Warfarin (ng ml^{-1})		
	37.5	150	1500	37.5	150	1500
Inter-day						
mean	37.5	150	1560	37.7	151	1570
RSD (%)	6.9	3.0	3.9	7.2	3.8	4.0
RE (%)	0.0	0.0	+4.3	+0.5	+0.5	+4.6
n	36	36	35	36	36	36
Intra-day						
mean	38.0	144	1490	35.9	144	1500
RSD (%)	3.9	1.3	1.9	11.8	2.7	2.1
RE (%)	+1.4	−4.3	−0.56	−4.3	−4.3	0
n	6	6	6	6	6	6

Table 5
Stability of quality-control samples in human plasma

	S-Warfarin (ng ml^{-1})			*R*-Warfarin (ng ml^{-1})		
	37.5	150	1500	37.5	150	1500
Freeze–thaw stability						
(*n* = 6)						
1st Cycle	37.1 (6.7)	155 (0.9)	1600 (3.4)	36.5 (6.7)	157 (2.2)	1610 (3.6)
2nd Cycle	38.7 (5.2)	155 (1.7)	1610 (1.6)	38.4 (3.3)	154 (1.6)	1610 (1.7)
As % of 1st cycle	104	100	101	105	98	100
3rd Cycle	37.6 (5.5)	152 (3.6)	1620 (0.9)	38.0 (3.3)	150 (4.6)	1620 (0.8)
As % of 1st cycle	101	98	101	104	96	101
Benchtop stability						
(*n* = 6) 0 h	36.9 (7.3)	152 (1.6)	1610 (1.4)	39.6 (5.0)	154 (1.7)	1610 (1.3)
2 h	36.7 (5.0)	153 (1.6)	1590 (1.8)	40.3 (6.3)	156 (1.2)	1590 (1.8)
As % of 0 h	99	101	99	102	101	99
On-system stability						
(*n* = 3) 0–80 min	39.8 (3.2)	155 (1.3)	1630 (1.6)	40.0 (3.1)	150 (2.3)	1640 (1.6)
33 h	38.0 (3.0)	158 (2.7)	1620 (1.1)	38.0 (4.1)	159 (1.3)	1630 (1.1)
As % of 0–80 min	95	102	99	95	106	99
Re-injection stability						
(*n* = 6) Initial	38.0 (3.7)	151 (1.5)	1590 (0.6)	38.7 (4.5)	154 (1.4)	1590 (0.7)
22 h	35.9 (4.3)	143 (2.5)	1460 (3.5)	37.0 (5.2)	145 (2.3)	1470 (3.4)
As % of initial	94	95	92	96	94	92
Sample storage stability						
(*n* = 6) Initial	38.0 (3.7)	151 (1.5)	1590 (0.6)	38.7 (4.5)	154 (1.4)	1590 (0.7)
22 days	38.0 (3.9)	144 (1.3)	1490 (1.9)	35.9 (11.8)	144 (2.7)	1500 (2.1)
As % of initial	100	95	94	93	94	94

RSDs are indicated in parentheses.

benchtop), chromatography (on-system and re-injection), and sample storage were tested and established. The data are presented in Table 5.

QCs were subjected to various cycles of freezing and thawing. The samples were then analysed. Table 5 shows that both enantiomers were stable after multiple cycles of freezing and thawing. After two cycles of freeze–thaw, the values of QC samples were 100–104% of that for one cycle for *S*-warfarin, and 98–105% for *R*-warfarin. After three cycles of freeze–thaw, the values of QC samples were 98–101% of that for one cycle for *S*-warfarin, and 96–104% for *R*-warfarin.

Benchtop stability after 2 h at room temperature was 99–101% for *S*-warfarin and 99–102% for *R*-warfarin compared to the corresponding values of normal time. The on-system stabilities after 33 h were 95–102% and 95–106% for *S*-warfarin and *R*-warfarin, respectively, compared to the original time. Samples re-injected after 22 h were 92–95% and 92–96% of the original corresponding values for *S*-warfarin and *R*-warfarin, respectively. Sample storage stability was tested after QCs were stored in a −20°C freezer for at least 22 days. The values of the stored samples were 94–100% and 93–94% of the corresponding values at the original assay for *S*-warfarin and *R*-warfarin, respectively.

We tested the stability of the stereoisomers against interconversion during the extraction step and chromatograph. Samples containing only one of the enantiomers were prepared. Due to the very limited quantities of the pure enantiomers available, the exact concentrations for the single enantiomers were not determined. This was not an issue because the purpose of this test was to determine whether there was interconversion of enantiomers. Table 6 shows that there was no enantiomer interconversion during extraction or chromatography since none of the unspiked enantiomer was detected after sample extraction and upon re-injection after 24 h on system.

Table 6
Extraction and on-system stability of single enantiomer

S-Warfarin			
0–100 min mean conc. (ng ml^{-1})		24-h mean conc. (ng ml^{-1})	
S-Warfarin	*R*-Warfarin	*S*-Warfarin	*R*-Warfarin
71.3	ND*	65.7	ND*
70.8	ND	70.4	ND
73.9	ND	69.3	ND
66.7	ND	68.4	ND
Mean 70.7		68.4	
RSD (%) 4.2		2.9	
n 4		4	

R-Warfarin			
0–100 min mean conc. (ng ml^{-1})		24-h mean conc. (ng ml^{-1})	
S-Warfarin	*R*-Warfarin	*S*-Warfarin	*R*-Warfarin
ND*	100.9	ND*	109.4
ND	106.0	ND	105.1
ND	111.8	ND	99.9
ND	101.3	ND	101.6
Mean	105.0		104.0
RSD (%)	4.9		4.0
n	4		4

*ND = Not detected.

Column variability

Batch-to-batch β-cyclodextrin column variability was observed. Figure 4 shows the chromatograms for each column from different batches. On columns A, B and C, good separation of *S*- and *R*-warfarin, metabolites, and I.S. was achieved, and no interference was observed from the plasma matrix. On column D, the overall separation and column efficiency was inferior, and a moderate interference peak from the matrix also partially coeluted with *S*-warfarin. The batch-to-batch β-cyclodextrin column variability was also observed for the enantioselective separation of *S*- and *R*-ibuprofen [15]. Three validation curves were independently performed on columns A–C. Inter-column precision and accuracy of *S*- and *R*-warfarin standards are shown in Table 7. Quantitative results for quality control at a concentration of 1500 ng ml^{-1} are shown in Table 8. Both tables show a good agreement among the results obtained on three columns. The RSD values and the mean results in Table 8 were analysed by an *F*-test and a Student's *t*-test ($P = 0.05$) [16]. In no instance were the *F*-test or the Student's *t*-test significant.

Conclusions

The objective of this study was to develop a chiral separation method that was simple, rugged, and applicable for the analysis of a large number of samples in a pharmacokinetic study. The method was validated to meet the pharmaceutical industry guidelines according

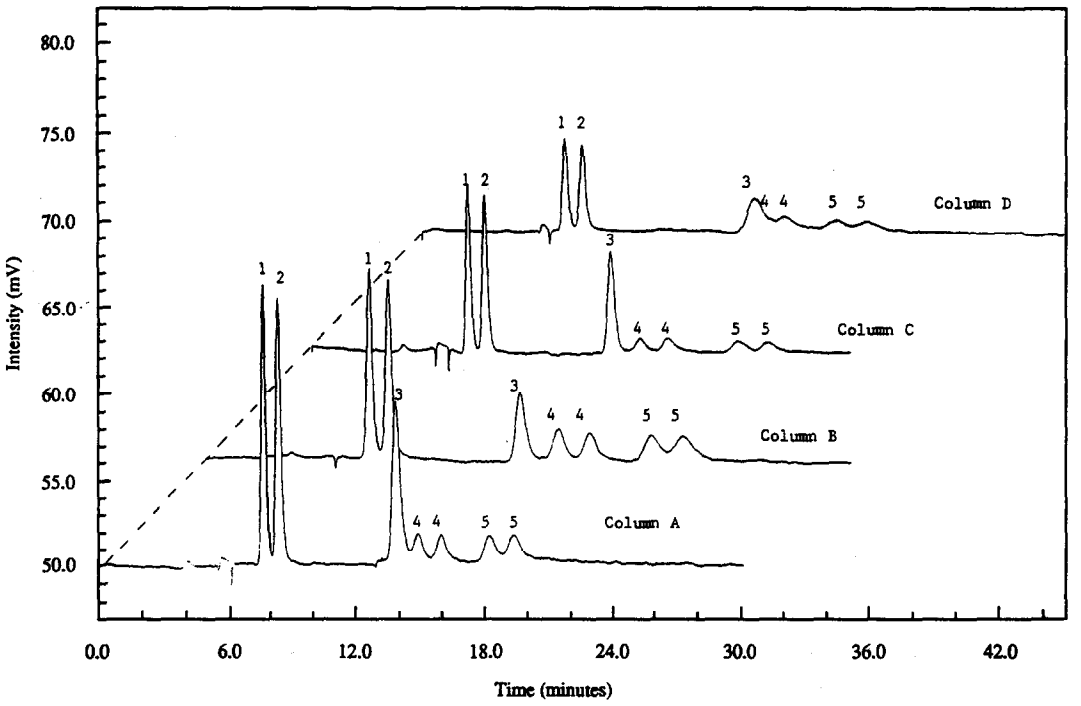

Figure 4
Column variability. Peak identification: 1, *S*-warfarin; 2, *R*-warfarin; 3, I.S.; 4, 6-hydroxywarfarin; 5, 7-hydroxywarfarin.

Table 7
Inter-column precision and accuracy at *S*- and *R*-warfarin standards

	Standard conc. (ng ml^{-1})						
	25	50	100	250	500	1000	2500
S-Warfarin							
Column A	27.7	45.1	103	252	487	980	2530
Column B	24.8	55.9	92.4	254	487	983	2530
Column C	25.6	48.5	95.5	238	489	998	2530
Mean	26.0	49.8	97.0	248	488	987	2530
RSD (%)	5.8	11.1	5.6	3.6	0.2	1.0	0
RE (%)	+4.17	−0.35	−2.96	−0.78	−2.46	−1.29	+1.23
n	3	3	3	3	3	3	3
R-Warfarin							
Column A	27.7	46.3	98.9	254	489	985	2520
Column B	25.6	50.8	100	251	481	976	2540
Column C	24.4	46.5	98.4	236	490	999	2530
Mean	25.9	47.8	99.2	247	487	987	2530
RSD (%)	6.5	5.3	1.0	4.0	1.0	1.2	0.4
RE (%)	+3.53	−4.31	−0.79	−1.22	−2.66	−1.32	+1.30
n	3	3	3	3	3	3	3

to the 1990 Conference Report [17], with additional tests on enantiomer interconversion through processing and chromatograph. No degradation and transformation of enantiomers were observed during the extraction and injection process. The β-cyclodextrin column maintained integrity after more than 1000 injections indicated by almost identical resolution of *S*- and *R*-warfarin and their retention times. When compared to other methods, the advantages of this method lie in its simplicity, short run time, column stability

Table 8
Inter-column precision and accuracy of *S*- and *R*-warfarin quality control

	S-Warfarin (1500 ng ml^{-1})			*R*-Warfarin (1500 ng ml^{-1})		
	Column A	Column B	Column C	Column A	Column B	Column C
	1520	1500	1470	1520	1480	1470
	1480	1440	1540	1470	1410	1550
	1510	1510	1510	1510	1480	1520
	1520	1470	1480	1520	1440	1490
	1450	1450	1470	1440	1430	1470
	1510	1480	1480	1510	1450	1480
Mean	1500	1480	1490	1500	1450	1500
RSD (%)	1.9	1.9	1.9	2.2	1.9	2.1
RE (%)	−0.11	−1.67	−0.56	−0.33	−3.44	−0.22
n	6	6	6	6	6	6
Mean of mean		1490			1480	
RSD (%)		0.8			1.9	
RE (%)		−0.78			−1.33	
n		3			3	

and reproducibility, assay sensitivity, and above all, its reliability. This method is currently being used for drug interaction studies.

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