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Report

The Measurement of Warfarin Enantiomers in Serum Using Coupled Achiral/Chiral, High-Performance Liquid Chromatography (HPLC)

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An assay for the serum concentration of the enantiomers of warfarin, *R*-warfarin and *S*-warfarin, has been developed using a bovine serum albumin chiral stationary phase (BSA-CSP) coupled to a Pinkerton internal-surface reverse-phase (ISRP) achiral column. The ISRP column is used to separate *R,S*-warfarin from the serum components and warfarin metabolites and to quantitate the total *R,S*-warfarin concentration. The eluent containing *R,S*-warfarin is then selectively transferred to the BSA-CSP, where the enantiomers are stereochemically resolved ($\alpha = 1.19$) and the enantiomeric composition is determined. This system is sensitive and accurate, does not require extensive precolumn manipulations, and can be automated for use in large-scale clinical studies.

KEY WORDS: warfarin enantiomers; achiral/chiral high-performance liquid chromatography (HPLC); enantioselective HPLC; serum levels.

INTRODUCTION

Warfarin [3-(α-acetonylbenzyl)-4-hydroxyxoumarin; Fig. 1] is a chiral molecule that is administered as a racemic mixture. The enantiomorphs of warfarin, (−)-*S*-warfarin (S-War) and (+)-*R*-warfarin (R-War) differ in their potency, rate of elimination, and plasma protein binding. For example, in humans, the hypoprothombinemic effect of S-War is two to five times greater than that of R-War (1–3), S-War is eliminated from the body about two to four times more rapidly than R-War (1,4,5), and S-War is more strongly bound to human albumin than the *R*-isomer (6,7).

The pharmacological and pharmacokinetic differences between the enantiomers of warfarin raise the issue of the rapid and accurate determination of the serum concentration of these isomers. A number of stereoselective assays have been developed to accomplish this. The approaches include the mass spectral analysis of a pseudoracemic mixture of ^{12}C-R-War and ^{13}C-S-War (4,8), the use of stereospecific antibodies (9), and the high-performance liquid chromatographic (HPLC) separation of diastereomeric derivatives (10,11). The first two of these assays involve the use of highly specialized and not readily available materials and the latter assay contains lengthy extraction and derivatization procedures.

We have attempted to shorten and simplify the stereoselective assay of R-War and S-War through the use of enantioselective HPLC. The stereochemical resolution of underivatized R-War and S-War has been reported using an HPLC chiral stationary phase composed of immobilized bovine serum albumin (BSA-CSP) (12). While this separation was adequate for the analysis of warfarin standards, it could not be directly used with serum samples due to interference from serum components and warfarin metabolites. To overcome this problem, the BSA-CSP was coupled to an achiral precolumn—a Pinkerton internal-surface reverse-phase (ISRP) column (13). In this system, which is diagramed in Fig. 2, the ISRP column is used to separate R,S-War from the serum components and warfarin metabolites and to quantitate the total warfarin concentration. The eluent containing R,S-War is then selectively transferred to the BSA-CSP, where R- and S-War are stereochemically resolved and the enantiomeric composition is determined. This system is accurate, does not require extensive precolumn manipula-

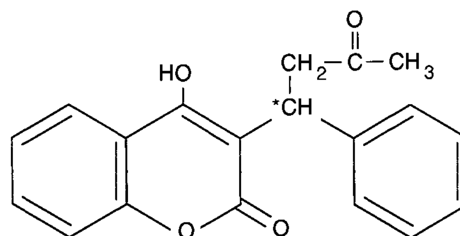

Fig. 1. The structure of warfarin.

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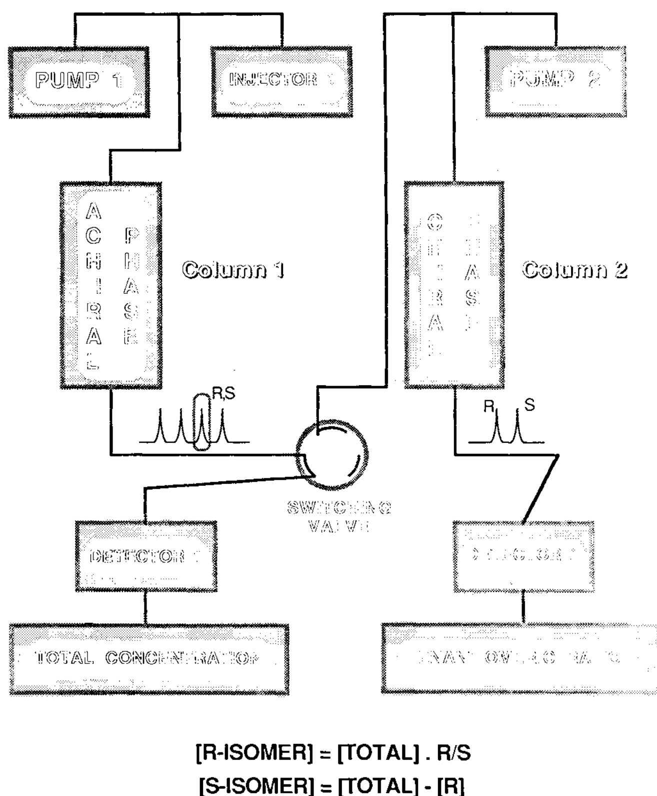

Fig. 2. A diagram of the coupled column system used in this study.

tions, and can be automated for use in large-scale clinical studies.

EXPERIMENTAL

Apparatus. The achiral chromatography was performed with a modular liquid chromatograph composed of a Beckman 110B solvent delivery module pump (Beckman Instruments, Inc., Houston, Tex.), a Spectra-Physics SP 8480XR scanning UV detector set at 225 nm (Spectra-Physics, Santa Clara, Calif.), a Fisher Recordall Series 5000 recorder (Fisher Scientific, Pittsburgh, Pa.), a Rheodyne 7125 injection valve (Rainin Instruments, Co., Inc., Woburn, Mass.), and a Pinkerton ISRP HPLC column (5 cm × 4.6-mm i.d., Regis Chemical Co., Morton Grove, Ill.).

The enantioselective chromatography was performed with a modular liquid chromatograph composed of a Beckman 110B solvent delivery module pump, a Spectra-Physics Spectraflow 980 fluorescence detector (excitation, 310 nm; emmission, 370 nm), a Shimadzu C-R6A Chromatopac integrator (Shimadzu Scientific Instruments, Inc., Columbia, Md.), and a commercially packed chiral-phase HPLC column composed of bovine serum albumin immobilized on 10-μm spherical silica (Resolvosil, Rainin Instruments Co., Inc.). The column temperature was maintained within ±0.1°C of the desired setting with a Forma Scientific Model 2006 circulating water bath (VWR Scientific, Chicago, Ill.) and an Alltech HPLC column water jacket (Alltech, Deerfield, Ill.).

The two systems were connected through a Rheodyne Model 7010 switching valve equipped with a pneumatic actuator and a 1-ml sample loop (Fig. 2). When the eluent fraction from column 1 containing *R,S*-warfarin was detected in detector 1, the switching valve was rotated and the eluent flow diverted to the sample loop on column 2. After 60 sec the switching valve was rotated and the eluent fraction containing *R,S*-warfarin was injected onto column 2.

Chemicals. Racemic warfarin was purchased from Sigma Co. (St. Louis, Mo.). The warfarin enantiomers, R-War and S-War, were prepared using the method described by West *et al.* (14). The warfarin metabolites 4′-hydroxywarfarin, 6-hydroxywarfarin, 7-hydroxywarfarin, and 8-hydroxywarfarin and warfarin alcohols were provided by Professor W. F. Trager, University of Washington, Seattle. Trichloroacetic acid was purchased from J. T. Baker Chemical Co. (Phillipsburg, N.J.) and the HPLC-grade 1-propanol was purchased from American Scientific (Muskegon, Mich.). Benoxaprofen was obtained from Eli Lilly and Co. (Indianapolis, Ind.). The other chemicals were reagent grade and used as purchased.

Chromatographic Conditions. The mobile phase used in the achiral chromatography was composed of 0.2 *M* phosphate buffer (pH 6.5):1-propanol (99:1). The chromatography was carried out at room temperature with a flow rate of 1 ml/min.

The mobile phase used in the enantioselective chromatography was composed of 0.2 *M* phosphate buffer (pH 7.5):1-propanol (97:3) modified with 3 m*M* trichloroacetic acid. The chromatography was carried out at 30°C with a flow rate of 1 ml/min.

Sample Preparation. A 7-μl aliquot of the internal standard (benoxaprofen, 206 μg/ml in 0.2 *M* phosphate buffer, pH 10.0) was added to a 100-μl serum sample. This was followed by the addition of 1 ml acetonitrile. The mixture was vortexed for 1 min, then centrifuged for 5 min, and the supernatant was collected and dried under a stream of nitrogen. The resulting residue was reconstituted in 100 μl of phosphate buffer (0.2 *M*, pH 10.0) and injected onto the chromatographic system.

Standard Curves. A standard curve was constructed by adding a known amount of R,S-War to pooled serum obtained from the blood bank of St. Jude Children's Research Hospital. Samples containing total warfarin concentrations of 1.25, 2.5, 5.0, 10.0, and 20 μg/ml were used to prepare the standard curve. Six determinations were obtained at each concentration. R,S-War concentrations of 25 ng/ml can be detected and reliably quantified.

Clinical Samples. The clinical samples analyzed in this study were obtained from an investigation of the effect of cimetidine on the pharmacokinetics of warfarin. The subjects in this study received a daily dose of warfarin (maximum, 15 mg/day) and various doses of cimetidine. Blood samples were collected at specified times, then allowed to clot, and the serum was harvested. The serum was then frozen at −20°C until analyzed.

RESULTS AND DISCUSSION

Achiral Chromatography

The results from the chromatography of warfarin, the major warfarin metabolites, and benoxaprofen on the ISRP column are presented in Table I. These results indicate that

Table I. Chromatographic Results

Compound	ISRP column,[a] k'[b]	BSA column[c]		
		k'_1[d]	k'_2[e]	α[f]
Warfarin	2.75	6.40	7.64	1.19
4′-Hydroxy warfarin	1.56	4.20	4.20	1.0
6-Hydroxy warfarin	1.69	4.90	4.90	1.0
7-Hydroxy warfarin	1.09	5.60	7.20	1.29
8-Hydroxy warfarin	2.16	6.30	7.30	1.16
Warfarin alcohols	2.28	5.10	5.90	1.16
Benoxaprofen	4.75			

[a] Mobile phase, 0.2 *M* phosphate buffer (pH 6.5):1-propanol (99:1); flow rate, 1 ml/min; temperature, ambient.
[b] Capacity factor.
[c] Mobile phase, 0.2 *M* phosphate buffer (pH 7.5):1-propanol (97:3) modified with 3 m*M* trichloroacetic acid; flow rate, 1 ml/min; temperature, 30°C.
[d] Capacity factor of first eluted enantiomer.
[e] Capacity factor of second eluted enantiomer.
[f] Stereochemical selectivity.

Table II. Precision and Accuracy

Achiral	Sample A	Sample B
Within day (N = 6)		
Spiked concentration (μg/ml)	1.25	10.00
Mean determined concentration (μg/ml)	1.24	11.10
CV (%)	3.74	4.00
Between day (N = 6)		
Spiked concentration (μg/ml)	1.25	12.50
Mean determined concentration (μg/ml)	1.25	11.40
CV (%)	2.60	1.20
Chiral	***S*-Warfarin**	***R*-Warfarin**
Between day (N = 7)		
Area percentage	50.0	50
Mean determined area percentage	55.6	44.4
CV (%)	0.9	1.0

warfarin can be separated from its major metabolites using the chromatographic conditions described in this paper. Representative chromatograms of 100 μl of blank serum and 100 μl of serum spiked with *R*,*S*-warfarin (10 μg/ml) and benoxaprofen (7 μl of a 206 μg/ml solution) are presented in Figs. 3A and B, respectively.

The standard curve for *R*,*S*-warfarin was linear over the range investigated. The equation describing the curve was $y = 0.714x + 0.013$, with a correlation coefficient of 0.999. The within-day and between-day measurements were done with 1.25 and 10.0 μg/ml samples of warfarin (within day) and 1.25 and 12.5 μg/ml samples of warfarin (between day). The precision and accuracy are summarized in Table II. The extraction efficiency averaged 95.1% over a 100-fold concentration range (Table III).

The chromatogram resulting from the analysis of a serum sample from one of the subjects in the clinical study is presented in Fig. 3C. The peaks that elute between warfarin (I) and benoxaprofen (II) have not been identified.

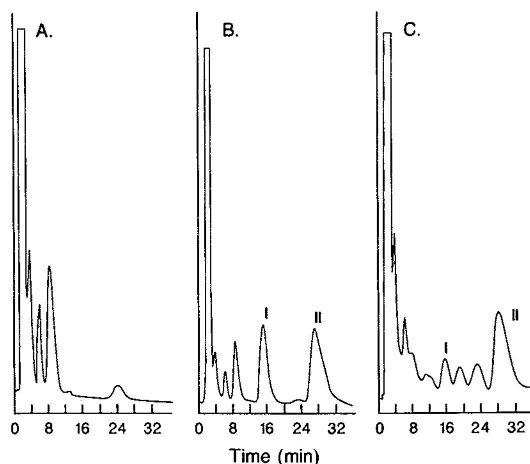

Fig. 3. Representative chromatograms on the achiral ISRP column for (A) an extracted blank serum sample, (B) a serum sample spiked with 10 μg/ml *R*,*S*-warfarin and internal standard, and (C) a serum sample from the clinical study. I, *R*,*S*-warfarin; II, benoxaprofen (internal standard).

Enantioselective Chromatography

The results from the chromatography of warfarin and the major warfarin metabolites on the BSA-CSP are presented in Table I. The elution order of (*S*)-warfarin and (*R*)-warfarin was determined using unequal mixtures of the resolved enantiomers. Under the chromatographic conditions employed in this study, (*S*)-warfarin elutes first, with a capacity factor (k') of 6.40, while the k' for (*R*)-warfarin is 7.64. The observed stereochemical selectivity (α) was 1.19 and the stereochemical resolution factor (R_s) was 1.05. It is of interest to note that the enantiomers of 7-hydroxywarfarin and 8-hydroxywarfarin were also resolved (α = 1.29 and 1.16, respectively), as were the diastereomeric warfarin alcohols (α = 1.16). However, 4′-hydroxywarfarin and 6-hydroxywarfarin were not resolved using these chromatographic conditions.

Representative chromatograms of 100 μl of blank serum and 100 μl of serum spiked with *R*,*S*-warfarin (5 μg/ml) are presented in Figs. 4A and B, respectively. The chromatogram resulting from the analysis of a serum sample from one of the subjects in the clinical study is presented in Fig. 4C.

The determination of the enantiomeric composition of the warfarin samples is based upon the relative peak areas of the two isomers. The precision and accuracy of this measurement were determined using a stock serum sample containing 5 μg/ml of *R*,*S*-warfarin (i.e., 2.5 μg/ml of each enan-

Table III. Percentage Recovery from Serum

Sample (μg/ml)	Percentage recovery[a]
0.5	95.3
5.0	95.7
50.0	94.2

[a] Average of six determinations.

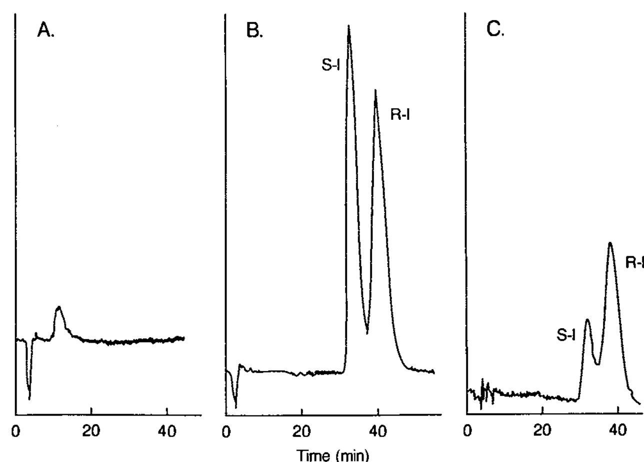

Fig. 4. Representative chromatograms on the chiral BSA-CSP column for (A) an extracted blank serum sample, (B) a serum sample spiked with 5 μg/ml *R,S*-warfarin, and (C) a serum sample from the clinical study. S-I *S*-warfarin; R-I *R*-warfarin.

tiomer). The mean determined area percentage for each enantiomer of a racemic mixture should be 50%. However, the experimentally determined areas for *S*-warfarin and *R*-warfarin were 55.6 and 44.4%, respectively (Table II). This difference is due to the incomplete chromatographic resolution of the two warfarin peaks and their shapes (Fig. 4). Since the ratio of the observed area percentage was not unity, a correction factor of 1.11 was used to calculate the actual percentage composition of each enantiomer. The observed area percentage of *S*-warfarin was divided by this factor, while the observed area percentage of *R*-warfarin was multiplied by this factor. This correction factor is applicable to a wide range of R/S ratios.

Clinical Samples

Nine clinical samples from a study of the interaction between warfarin and cimetidine were analyzed using this method and the results are presented in Table IV. The total concentration of *R,S*-warfarin ([R,S-War]) was first determined using the ISRP column. A second analysis was then performed using the coupled ISRP and BSA-CSP columns to determine the area percentage of *S*-warfarin (%S-War) and *R*-warfarin (%R-War). The total amounts of *S*-warfarin and *R*-warfarin were determined using the following equations:

$$\text{total } S\text{-warfarin} = [\text{R,S-War}] \times (\%\text{S-War})$$
$$\text{total } R\text{-warfarin} = [\text{R,S-War}] \times (\%\text{R-War})$$

The results of this study will be reported elsewhere.

CONCLUSION

A number of HPLC chiral stationary phases use aqueous mobile phases and are able to resolve stereochemically enantiomeric molecules without procolumn derivatization. These phases include the BSA-CSP used in this study as well as chiral phases based on immobilized α-1-acid glycoprotein and cylcodextrin. These phases can be easily coupled to achiral reverse-phase precolumns, and the resulting systems used for the direct analysis of biological samples. The assay described in this paper is an example of this approach.

Table IV. The Serum Concentration of *S*-(−)- and *R*-(+)-Warfarin

Patient No.	Day	Hour	*S*-(−)-Warfarin (μg/ml)	*R*-(+)-Warfarin (μg/ml)
1	21	10	0.23	2.12
2	49	6	0.42	1.48
2	49	8	0.22	1.15
3	49	4	0.50	0.94
4	21	4	0.20	1.85
4	21	10	0.12	1.84
5	21	1	0.32	0.90
5	49	2	0.30	0.97
5	49	10	0.22	0.62

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REFERENCES

1. A. Breckenridge, M. O. Rue, H. Wesseling, R. J. Lewis, and R. Gibbons, *Clin. Pharmacol Ther.* 15:424–430 (1974).
2. J. N. Eble, D. B. West, and K. P. Link. *Biochem. Pharmacol.* 15:1003–1006 (1966).
3. R. A. O'Reilly. *Clin. Pharmacol. Ther.* 16:348–354 (1974).
4. C. Hignite, J. Uetrect, C. Tschanz, and D. Azarnoff. *Clin. Pharmacol. Ther.* 28:99–105 (1980).
5. D. S. Hewick and J. McEwan. *J. Pharm. Pharmacol.* 25:458–465 (1973).
6. E. M. Sellers and J. Koch-Weser. *Pharmacol Res. Comm.* 7:331–334 (1975).
7. J. H. M. Miller and G. A. Smail. *J. Pharm. Pharmacol.* 29:Suppl. 33P (1977).
8. W. N. Howald, E. D. Busch, W. F. Trager, R. A. O'Reilly, and C. H. Motely. *Biomed. Mass Spectr.* 7:35–40 (1980).
9. C. E. Cooke, N. H. Ballentine, T. B. Saltzman, and C. R. Tallent. *J. Pharmacol. Exp. Ther.* 210:391–398 (1979).
10. C. R. Banfield and M. Rowland. *J. Pharm. Sci.* 72:921–924 (1983).
11. C. R. Banfield and M. Rowland. *J. Pharm. Sci.* 73:1392–1396 (1984).
12. S. Allenmark. *J. Liq. Chromatogr.* 9:425–442 (1986).
13. T. C. Pinkerton, T. D. Miller, S. E. Cook, J. A. Perry, J. D. Rateike, and T. J. Szczerba. *Biochromatography* 1:96–105 (1986).
14. B. D. West, S. Preis, C. H. Schroeder, and K. P. Link. *J. Am. Chem. Soc.*, 2676–2679 (1961).