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Sensitive stereospecific assay of warfarin in plasma: reversed-phase high-performance liquid chromatographic separation using diastereoisomeric esters of (−)-(1*S*,2*R*,4*R*)-*endo*-1,4,5,6,7,7-hexachlorobicyclo[2.2.1]-hept-5-ene-2-carboxylic acid

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ABSTRACT

A stereospecific reversed-phase high-performance liquid chromatographic (HPLC) assay for warfarin in plasma has been developed. The assay involves a rapid, simple clean-up procedure which separates warfarin from plasma constituents and warfarin metabolites. Warfarin enantiomers were assayed as their (−)-(1*S*,2*R*,4*R*)-*endo*-1,4,5,6,7,7-hexachlorobicyclo[2.2.1]hept-5-ene-2-carboxylic acid diastereoisomeric esters by reversed-phase HPLC. Excellent resolution of the diastereoisomers was achieved in less than 10 min. Sensitivity of the assay was approximately 5–10 ng/ml for each isomer.

INTRODUCTION

Oral anticoagulant treatment in Australia and in most countries involves the administration of racemic warfarin. Stereoselective metabolism, different potencies [1–3] and stereoselective drug interactions [4–7] of warfarin enantiomers suggest that the plasma drug concentration and anticoagulant effect relationship would be better described if the concentrations of both enantiomers rather than that of the total drug were measured. Recent studies showing favourable antithrombotic effects of racemic warfarin with doses as low as 1 mg daily [9,10] point towards the need for assays with lower limits of detection of the stereoisomers than were necessary for assays of total drug at the usual doses of 4–10 mg [8,10].

High-performance liquid chromatography (HPLC) of individual warfarin enantiomers in biological fluids has been accomplished via the synthesis of warfarin diastereoisomeric ester derivatives with N-carbobenzyloxy-L-proline followed by normal-phase HPLC [11,12]. The limit of detection was reduced below that using UV [11] by the use of fluorescence after post-column alkalinization of the eluent, which was necessary to convert non-fluorescent warfarin diastereoisomeric esters to fluorescent warfarin. This was achieved by aminolysis using *n*-butylamine–methanol (1:1) which was miscible with the non-polar mobile phase. Fluorescence detection reduced the detection limit to 50–100 ng/ml of plasma for each enantiomer. Recently, a coupled achiral–chiral HPLC system was reported for the detection of warfarin enantiomers in human serum [13] and a detection limit as low as 25 ng/ml was obtained although no estimate of the accuracy and precision at concentrations below 1.25 μg/ml were presented.

Several non-stereoselective HPLC assays have

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been reported for warfarin in biological fluids [14–16], the most sensitive being a reversed-phase HPLC assay [16] which utilized fluorescent detection with post-column alkalinization to enhance warfarin fluorescence. Adequate sensitivity, accuracy and precision for total plasma warfarin concentrations were reported in the therapeutic range (10–20 ng/ml), the limit of detection being 2–4 ng/ml.

The present study investigates the use of new diastereoisomeric derivatives of warfarin which could be separated by reversed-phase HPLC and hydrolysed post-column to provide optimal fluorescence and assay sensitivity. *endo*-1,4,5,6,7,7-Hexachlorobicyclo[2.2.1]hept-5-ene-2-carboxylic acid (HCA) is a readily prepared resolvable bicyclic carboxylic acid [17]. Separation of diastereoisomers prepared with (+)-HCA allowed the successful preparation and analysis of enantiomers of dihydrodiol and tetrahydrodiol derivatives of the carcinogens 7-methylbenz[*c*]acridine and dibenz[*a*,*j*]acridine [18].

The (−)-(1*S*,2*R*,4*R*)-HCA isomer is used in this study to prepare (−)HCA-warfarin diastereoisomers which are easily separated by reversed-phase HPLC. This is the first reported use of HCA in a stereospecific analysis of a therapeutic compound in biological fluids.

EXPERIMENTAL

Materials

Warfarin sodium was received as a gift from Dupont (Wilmington, DE, USA). HPLC-grade acetonitrile, diethyl ether, methanol and hexane (Mallinkrodt, Paris, KY, USA), N,N′-dicyclohexylcarbodiimide (DCC) and N-cyclohexyl-N′-[2-(1-methylmorpholin-1-yl)ethyl]-carbodiimide-*p*-toluenesulphonate (CMC) (Fluka, Buchs, Switzerland), TLC-grade silica gel H (Merck, Darmstadt, Germany) and racemic *p*-chlorowarfarin [3-(2-acetonyl-*p*-chlorobenzyl)-4-hydroxycoumarin] (Sigma, St. Louis, MO, USA) were obtained from commercial sources. (−)-(1*S*,2*R*,4*R*)-HCA was prepared as described [17]. Enantiomers of *p*-chlorowarfarin and warfarin (greater than 99% optically pure) were prepared from racemic *p*-chlorowarfarin and warfarin, respectively, by separation and hydrolysis of the (−)-HCA esters [19]. (−)-HCA may be purchased by contacting the authors. Distilled water and all HPLC solvents were filtered by a 10-μm vacuum filter prior to use.

Extraction procedure

Methanolic solutions of the internal standard, (+)-*p*-chlorowarfarin (50–500 μl of 0.1 mg/ml for higher warfarin levels or 40–500 μl of 1 μg/ml for lower levels), were dried in test tubes under a stream of nitrogen before adding citrated plasma (1 ml), 1 *M* hydrochloric acid (100 μl) and freshly distilled diethyl ether (5 ml). The mixture was vortex-mixed for 30 s and centrifuged at 1000 *g* for 5 min. The ether layer was separated and dried at 40°C under a stream of nitrogen.

Single-use clean-up chromatographic columns consisting of silica gel H (250 mg) packed under vacuum in Pasteur pipettes were washed with distilled diethyl ether (3 × 1 ml) and then with loading solution [hexane–distilled diethyl ether (4:1), 3 × 1 ml] prior to use.

The dried plasma extract was reconstituted with loading solution (1 ml) and passed through the silica column. After washing the column with loading solution (2 × 1 ml), it was eluted with distilled diethyl ether (1 ml). The first 200 μl of eluate were discarded and the remainder was collected and dried on a heat bed at 40°C under a stream of nitrogen and derivatized.

Derivatization

(−)-HCA (15 μl, 50 mg/ml in acetonitrile–water, 25:1) and a solution (10 μl) of either DCC (100 mg/ml) or CMC (300 mg/ml) in acetonitrile–water (25:1) were added to the dried extract. The reaction was complete in 10 min using DCC and in 1 h using CMC.

A 20-μl injection of the reaction mixture was introduced onto the HPLC column within 4 h of derivatization.

High-performance liquid chromatography

The mobile phase, acetonitrile–distilled water (80:20), was pumped using an Altex 110A HPLC pump at a flow-rate of 1.6 ml/min through a precolumn (15 mm × 4 mm I.D.), then a Merck HPLC column (250 mm × 4 mm I.D.) both containing 10-μm LiChrosorb C_{18} reversed-phase

packing. A second HPLC pump was used to mix the column effluent with 0.2 *M* aqueous sodium hydroxide pumped at 0.5 ml/min via a T-piece. The resulting solution was passed through a 1-m reaction coil prior to detection using a Perkin Elmer fluorescence spectrophotometer with λ_{exc} = 313 nm. A cut-off filter allowed all fluorescence with wavelength greater than 370 nm to be measured. The quantities of (+)-(*R*)- and (−)-(*S*)-warfarin were interpolated from a six- to nine-point standard curve of peak-height ratio *versus* concentration constructed daily by spiking plasma with various quantities of racemic warfarin and (+)-chlorowarfarin.

Recovery

Using the assay of Erlandsson *et al.* [20], the recovery of warfarin isomers from the ether extraction step was determined from peak heights of *R*(+)- and *S*(−)-warfarin resulting from three plasma samples (1 ml) that had been spiked with 10 μg of racemic warfarin, compared with peak heights from the direct injection of 10 μg of racemic warfarin. The recovery in the complete assay was determined by comparing the peak heights resulting from the extraction, clean-up and derivatization of three plasma samples (1 ml) spiked with 40 ng of racemic warfarin to the peak heights resulting from derivatization and HPLC of 40 ng of racemic warfarin.

Gas chromatographic–mass spectrometric (GC–MS) examination of plasma

Aliquots (1.0 ml) of pooled plasma samples from three patients who had been stabilised on racemic warfarin for a period greater than three weeks were extracted with diethyl ether. GC–MS was conducted directly on the dried ether extract and on the final residue after the silica clean-up stage.

The GC column was a BP1 bonded-phase fused-silica capillary (SGE, Melbourne, Australia; 9 m × 0.2 mm I.D., 0.25 μm film thickness). The column temperature programme began at 150°C and rose to 300°C at 25°C/min, with the injection port at 250°C and the transfer line at 300°C. The carrier gas was helium with a flow-rate of 2 ml/min.

A Finnigan TSQ triple-stage quadrupole GC–MS–MS system, operated in Q_1 mode with negative-ion chemical ionization was used with methane as the reagent gas and a source pressure of 133 Pa. Electron beam energy was 100 eV. The scan range was 100–380 dalton with approximately 0.5 scan per second.

RESULTS AND DISCUSSION

The ether extraction stage gave average (± S.D.) recoveries of 103 ± 2 and 101 ± 2% for (+)-(*R*)- and (−)-(*S*)-warfarin, respectively. The chromatographic clean-up step with silica was introduced to remove warfarin metabolites and interfering peaks, and caused the majority of losses. Average (± S.D.) recovery from the extraction and clean-up procedure was 69 ± 9 and 67 ± 10% for (+)-(*R*)- and (−)-(*S*)-warfarin, respectively (40 ng/ml).

In contrast to the synthesis of warfarin diastereoisomers with carbobenzyloxy-L-proline which required catalysts [11], warfarin reacted with (−)-HCA using either DCC or CMC as condensing agents in the absence of catalysts. Using DCC, the reaction was complete within 18 min, but the reaction mixture contained a precipitate of N,N′-dicyclohexylurea, which is relatively insoluble in a wide variety of aqueous and organic solvents [11]. This necessitated frequent replacement of the pre-column. Also, at very low levels of warfarin, interfering peaks occurred with approximately the same retention time as warfarin diastereoisomers when DCC was used.

The carbodiimide reagent, CMC, and its condensation products, an N-cyclohexyl-N′-2-(1-methyl-1-morpholin-1-yl)ethylurea salt, are water-soluble allowing a simpler method of preparation of warfarin diastereoisomeric derivatives for reversed-phase HPLC analysis. The addition of water (4%) to the acetonitrile optimised the solubility of both CMC and its condensation product.

Using a 300 mg/ml CMC solution, the time course of formation of diastereoisomeric esters from racemic warfarin solution was followed over a period of 24 h. The reaction was virtually complete in 40–60 min with 97% of warfarin derivatised. The rates of formation of each diastereoisomer were very similar and after 1 h there

TABLE I

ACCURACY AND PRECISION AT HIGHER CONCENTRATION (0.1–5 μg/ml)

Values in parentheses are coefficients of variation (%).

Enantiomer concentration expected (μg/ml)	*n*	Concentration found (mean ± S.D.) (μg/ml)	
		(+)-(*R*)-Warfarin	(−)-(*S*)-Warfarin
1.25	3	1.288 ± 0.023 (1.8)	1.245 ± 0.022 (1.7)
0.50	4	0.500 ± 0.019 (3.8)	0.496 ± 0.016 (3.3)
0.25	4	0.251 ± 0.008 (3.2)	0.241 ± 0.007 (2.9)
Regression equation	9 points	$y = 0.0148 + 0.667x$ $r^2 = 0.9975$	$y = 0.0093 + 0.691x$ $r^2 = 0.9947$

was no further increase in the proportion of warfarin derivatives. After 4 h, hydrolysis of warfarin diastereoisomeric esters was insignificant while at 24 h about 80% of the diastereoisomers remained. The proportion of warfarin diastereoisomers was constant at about 50% of each (48–52%) indicating that selective hydrolysis had not occurred. At lower CMC concentrations (100 mg/ml) the reaction was not complete until 280 min. The CMC reagent in acetonitrile–water was found to be effective over a period of two to three months stored at room temperature.

Diastereoisomeric derivatives prepared from racemic warfarin and racemic chlorowarfarin separated well in both normal-phase and reversed-phase HPLC. There are distinct advantages with a reversed-phase rather than normal-phase [11,12] system in the warfarin assay, in terms of better peak shapes, greater separation of diastereoisomeric esters and more sensitive fluorescence detection of ionised warfarin achieved in polar aqueous mobile phase after post-column hydrolysis with sodium hydroxide.

In preliminary fluorescence spectrophotometric studies it was noted that fluorescence of warfarin in an aqueous environment was up to ten-fold higher than in a non-polar environment. Also hydrolysis rates obtained after mixing sodium hydroxide (>0.5 m*M*) with two volumes of HPLC mobile phase containing warfarin diastereoisomers solution could not be reliably estimated due to very rapid increase in fluorescence. At 0.5 m*M* sodium hydroxide, the half-time of the reaction was approximately 10 s, and for post-column hydrolysis the sodium hydroxide concentration was 0.2 *M* to ensure complete reaction.

Table I describes the accuracy and precision of the method using a nine-point standard curve constructed covering the range 0.1–5 μg/ml for each enantiomer. The accuracy and precision at those concentrations tested indicate a reliable and accurate assay procedure. Standard curves were linear and r^2 exceeded 0.99 in this range of concentrations. Chromatograms of blank plasma examined at appropriate spectrofluorimeter sensitivity are extremely clean. Fig. 1 is a chromatogram of a spiked plasma sample containing 0.25 μg/ml of each stereoisomer.

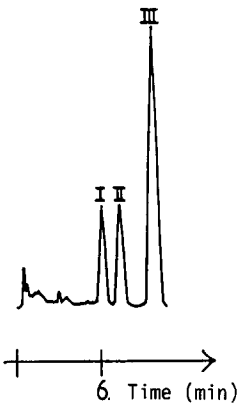

Fig. 1. Chromatogram of a derivatized extract from 1 ml of plasma spiked with 0.5 μg/ml racemic warfarin and 12 μg/ml *R*-chlorowarfarin. Sensitivity setting is 9 × 1. Peaks: I = *S*-warfarin ester; II = *R*-warfarin ester; III = *R*-chlorowarfarin ester.

TABLE II

ACCURACY AND PRECISION AT LOWER CONCENTRATIONS (2.0–40 ng/ml)

Values in parentheses are coefficients of variation (%).

Enantiomer concentration expected (ng/ml)	*n*	Concentration found (mean ± S.D.) (ng/ml)	
		(+)-(*R*)-Warfarin	(−)-(*S*)-Warfarin
10.0	4	10.86 ± 1.24 (11)	10.59 ± 1.19 (11)
5.0	4	5.49 ± 0.47 (8.6)	5.00 ± 0.47 (9.3)
2.0	4	2.24 ± 0.64 (28)	2.27 ± 0.25 (11)
Regression equation	7 points	$y = 0.0275 + 0.0717x$ $r^2 = 0.976$	$y = -0.0993 + 0.0839x$ $r^2 = 0.962$

Table II describes the accuracy and precision of the method using a seven-point standard curve covering the range 2–40 ng/ml for each enantiomer. Whilst demonstrating reasonable accuracy at those concentrations tested, higher variability is evident. At 5 and 10 ng/ml the coefficient of variation (C.V.) is approximately 10% which is probably the limit of quantitation. Chromatograms at this spectrofluorimeter sensitivity were very noisy as shown by the blank chromatogram (Fig. 2). Fig. 3 is a chromatogram resulting from the assay of spiked plasma with 5 ng/ml of each stereoisomer. The influence of plasma constituents on the variability at low levels was demonstrated by testing the precision of the assay of pure warfarin. The C.V. was determined at 2 ng each of pure warfarin enantiomers and found to be 3.0 and 1.5% for (+)-(*R*)- and (−)-(*S*)-warfarin, respectively. Chromatograms could not be improved by using an alkaline wash step prior to the acid extraction [12] and showed the presence of more fluorescent contaminants than when the procedure was omitted.

An extraction procedure based on the method of Fasco *et al.* [15], which uses commercially available reversed-phase Sep-Pak (Waters Assoc.) columns, was attempted. Derivatization of

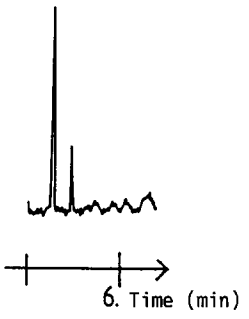

Fig. 2. Chromatogram of a derivatized extract from 1 ml of blank plasma at sensitivity setting 10 × 10. This level of sensitivity is used to measure warfarin enantiomers in the range 2–20 ng/ml.

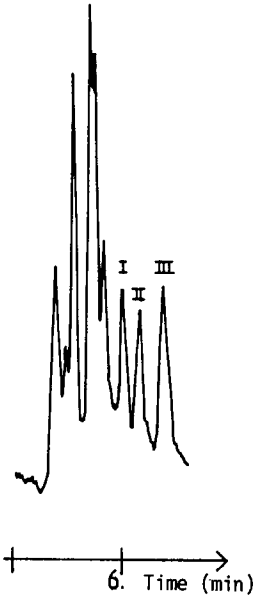

Fig. 3. Chromatogram of a derivatized extract from 1 ml of plasma spiked with 10 ng/ml racemic warfarin and 100 ng/ml internal standard. Sensitivity setting is 10 × 10. Peaks: I = *S*-warfarin ester; II = *R*-warfarin ester; III = *R*-chlorowarfarin ester.

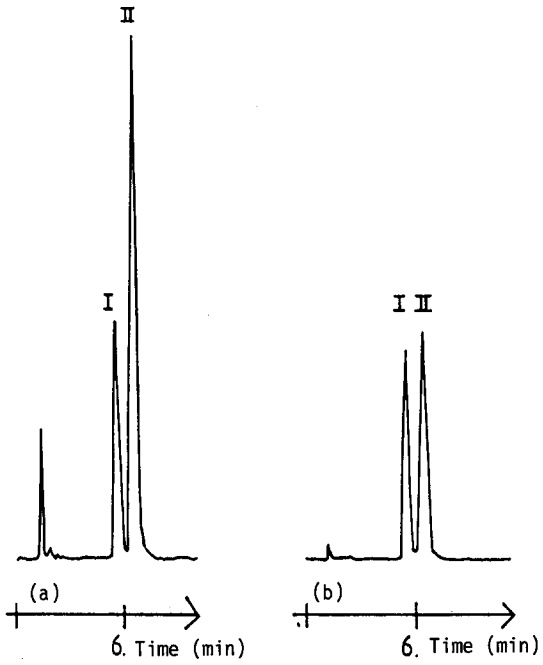

Fig. 4. Chromatograms of derivatized extract from 1-ml plasma samples of two patients (a and b) taking therapeutic and steady-state doses of racemic warfarin. Sensitivity setting 8 × 1. Peaks: I = *S*-warfarin ester; II = *R*-warfarin ester. No internal standard.

warfarin was not successful on the methanol–aqueous buffer eluate because the condensing agent(s) and diastereoisomeric ester products were destroyed by reaction with excess water. The eluate can be dried by the lengthy process of repeatedly adding methanol to the sample and evaporation with heat under a stream of nitrogen. However, chromatograms had interfering peaks which coeluted with the diastereoisomeric esters of warfarin and the internal standard.

Fig. 4 presents chromatograms resulting from the assay of 1-ml plasma samples taken from patients who had been taking racemic warfarin at dosage rates required to extend the prothrombin time into the therapeutic range. In each case the ratio of *R*- to *S*-warfarin was greater than 1 although only a small difference is evident in Fig. 4b. The observations are consistent with previous reports that the *S*-enantiomer is generally cleared from plasma more quickly than the *R*-enantiomer although large inter-individual variation in clearance ratios are reported [1–3]. These figures demonstrate chromatograms which are free from interfering peaks at the retention time of the internal standard.

In the present study quantification of warfarin metabolites was not attempted, their presence of metabolites being considered to be a potential interference. GC–MS experiments demonstrated that there was negligible interference in the assay due to warfarin metabolites.

The mass spectrum of warfarin showed a strong quasimolecular ion (Q) at *m/z* 307 resulting from deprotonation of warfarin. In addition there was a small but significant Q + 2 peak at *m/z* 309 due to the natural abundances of isotopes of carbon, hydrogen and oxygen. This must be taken into account in the identification of the metabolically formed diastereoisomeric warfarin alcohols [15] which have the same mass-to-charge ratio for their quasimolecular ions as the Q + 2 peak for warfarin.

The GC–MS analysis of the ether extract of plasma of patients receiving warfarin demonstrated warfarin (*m/z* 307) eluting at the GC elution time of 2 min 32 s with about 75% of the total ion current seen during the gas chromatogram. A search for dehydrowarfarin (*m/z* 305) yielded less than 0.2% of the total ion current. This metabolite was therefore not considered to be present in significant quantities. Warfarin alcohols (*m/z* 309) were identified as GC peaks, eluting at 2 min 39 s and at 3 min 2 s; the summed ion current of both peaks was approximately 15% of the total ion current. GC peaks corresponding to the phenylhydroxylated metabolites with *m/z* 323 were seen at 2 min 33 s, 3 min 6 s and 3 min 10 s. The summed ion current of these phenol peaks was approximately 2.5% of the total ion current. The metabolites were those expected and previously found in plasma of patients after oral warfarin [12].

The corresponding GC–MS profile of the ether extract of plasma which had been cleaned up by passage through the silica column yielded significant amounts of warfarin (*m/z* 307) with approximately 65% of the total ion current seen. Peaks corresponding to warfarin metabolites with *m/z* 305, 309 and 323 yielded less than 0.1% of the total ion current in each case. There was an ion at *m/z* 309 which was associated with spectra of material having the same retention time as warfarin

(2 min 32 s) with approximately 3% relative abundance. This corresponds to the relative abundance of the warfarin Q + 2 peak calculated above. The GC–MS analysis of extracted blank plasma yielded no significant peaks at any of the GC elution times associated with metabolites mentioned above. It was concluded that the chromatography on silica gel removed warfarin metabolites sufficiently to be confident that interference from them in the quantification of warfarin was not significant.

The formation of diastereoisomeric derivatives of warfarin with (−)-HCA and successful reversed-phase HPLC separation represents a significant improvement over earlier methods in the stereospecific analysis of warfarin in plasma. The absence of precipitation during the synthesis of (−)-HCA-warfarin diastereoisomers using CMC represents a significant improvement (from the HPLC maintenance point of view) over the synthesis of diastereoisomers with carbobenzyloxy-L-proline [11] where DCC was the only condensing agent to provide reliable results. The reversed-phase assay has a lower limit of detection than the normal-phase fluorescence detection assay of Banfield and Rowland [12]. That assay requires about 60 min of chromatography whilst the present reversed-phase HPLC assay provides a run time of less than 10 min. With aqueous sodium hydroxide as the post-column reagent hydrolysis occurs rapidly and a "bed-reactor" is not required.

The coupled achiral–chiral assay of Chu and Wainer [13] relies on the injection of a 1-ml eluent fraction from the initial achiral column (representing less than 100% of the total amount initially injected) onto an immobilized bovine serum albumin (BSA) column which separates warfarin enantiomers. The large injection volume (1.0 ml) resulted in significant peak broadening and, coupled with the effect of relatively broad peaks that are characteristic of BSA column separations [20,21], such an assay method is unlikely to prove more sensitive than the present method. While the BSA column procedure has a shorter preparation time than a procedure which requires derivatization, each stereoselective separation requires about 50 min on the BSA column.

The sensitivity of the present method is limited by interfering plasma constituents and not by the intrinsic fluorescence of warfarin in the mobile phase or by detector sensitivity. There is therefore scope for further improvement in the extraction and clean-up stages of the method to allow even lower limits of sensitivity in plasma and the possibility of extension of the technique to the assay of plasma ultrafiltrate and ultracentrifugate to allow free-level estimates.

It is possible that some drugs such as acidic fluorescent substances may interfere with the assay. Frusemide and indomethacin did not demonstrate interfering peaks; however, (+)-naproxen, or a derivative of it, co-elutes with (−)-HCA diastereoisomers of warfarin.

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