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Chiral CE separation of warfarin in albumin containing samples

Wai-Ping Yau, Eli Chan *

Department of Pharmacy, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260, Singapore

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Abstract

A capillary zone electrophoresis method with ultraviolet (UV) absorbance detection for the chiral separation of warfarin enantiomers using highly sulfated β-cyclodextrin (β-CD) was developed and optimized. Enantiomeric separation of warfarin was characterized by high resolution and efficiency. The optimized electrophoretic conditions were subsequently applied to the analysis of warfarin extracted from spiked human serum albumin samples. This assay showed acceptable precision, with linearity in the warfarin enantiomer concentration range of 0.1–25 mg/l. The limits of detection (LOD) and quantitation (LOQ) evaluated as warfarin enantiomer concentrations in the serum samples were 0.05 and 0.15 mg/l, respectively, for each warfarin enantiomer.

Keywords: Capillary zone electrophoresis; Chiral separation; Warfarin enantiomers; Highly sulfated β-cyclodextrin

1. Introduction

Warfarin [3-(α-acetonylbenzyl)-4-hydroxycoumarin], a synthetic coumarin derivative, is a widely used oral anticoagulant drug that is generally administered as the sodium salt commercially available as a racemic mixture composed of equal amounts of the *R*-(+) and *S*-(−)-enantiomers. These two optical isomers exhibit marked differences in pharmacokinetics and pharmacodynamics [1]. Warfarin is extensively bound to plasma protein (97.4–99.9%), primarily to albumin [2,3], with the *S*-isomer having greater binding affinity than the *R*-isomer [4]. In man, the *S*-isomer is about five times more potent [3], and is eliminated (by metabolism) more rapidly, than the *R*-isomer [3,4]. The concentration ratio of enantiomers in plasma, therefore, varies with time within an individual, and also between individuals. These differences play an important role in the anticoagulant effect of warfarin and the many clinically important drug interactions with warfarin that have a stereoselective basis [1–3].

In order to study the enantiomeric differences with respect to activity and pharmacokinetics, enantioselective analytical methods are required. Various analytical methods, namely gas chromatography (GC), high-performance liquid chro-

* Corresponding author. Tel.: +65-874-2932; fax: +65-779-1554.

E-mail address: phaelic@nus.edu.sg (E. Chan).

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matography (HPLC) and supercritical fluid chromatography (SFC), have been routinely used for separation of enantiomers [5]. Among them, HPLC has been used most commonly, with stereoselectivity being obtained by either chiral or achiral methods. The achiral methods were carried out via pre-column derivatization of the enantiomers of warfarin [6,7] and its major metabolites (6- and 7-hydroxywarfarin and warfarin alcohols) [6] using a chiral derivatising agent to form diastereomeric esters, followed by subsequent normal-phase HPLC [6] or reversed-phase HPLC [7]. The chiral methods involved the use of chiral stationary phases (CSPs), such as α_1-acid glycoprotein (α_1-AGP) [8] and β-cyclodextrin (β-CD) column [9], for direct column liquid chromatographic enantioseparation without prior diastereomeric derivatization. Although HPLC has been shown to be capable for enantioseparation of warfarin [6–9], this conventional method suffers a few drawbacks, such as lack of general applicability, high consumption of samples and expensive reagents, less robust and less cost-effective [5,10].

Capillary electrophoresis (CE) is one of the most recent and extensively developed alternate technique for the analysis of chiral and racemic drugs [5,11]. The great interest and rapid development of CE as a chiral separation technique over the last decade is due to its many advantages over the conventional chromatography methods, which include higher separation efficiency, greater flexibility, increased robustness, lower consumption of reagents and samples per run, shorter analysis time, greater simplicity in operation and applicability to a wider range of analytes, as compared with the older chromatographic techniques [5,10]. Chiral separations by CE are mostly achieved by employing the free solution capillary electrophoresis (FSCE) method [12]. Chiral separations by FSCE are obtained simply by adding a specific chiral selector to the background electrolyte (BGE) buffer. The chiral selector is able to recognize the enantiomers stereoselectively with different binding constants, resulting in chiral separation [10]. In published papers to-date, the various chiral selectors employed to the chiral separation and quantitation of warfarin enantiomers by direct chiral CE include human serum albumin [13], alkylglycoside surfactants [14], the glycopeptide antibiotic vancomycin [15], maltodextrins [5,16] and β-CD derivatives [17–22].

CDs, which are cyclic oligosaccharides of unique truncated cone shape prepared by enzymatic hydrolytic cleavage of starch, are the most popular electrophoretic chiral selectors. They are available in the native neutral forms (namely α-, β- and γ-CDs), as well as uncharged and charged derivatized forms such as methyl-β-CD (Me-β-CD), highly sulphated β-CD (HS-β-CD) and sulfobutyl ether β-CD (SBE-β-CD) [10,12,17–23]. For the optimization of the enantiomeric separation method of warfarin enantiomers in this study, the appropriate type of CD has to be selected. As reported by Gareil [17], warfarin forms inclusion complexes with β-CD like many other aromatic compounds, but no chiral resolution of warfarin enantiomers could be achieved using β-CD in FSCE. Instead, partial separation was obtained using Me-β-CD with a degree of substitution of 1.8 but the enantiomeric peaks were not well-resolved (resolution of less than 1) [17]. Besides the uncharged Me-β-CD [17], anionic SBE-β-CD [19,21] and the HS-β-CD [22] were also employed in the chiral separation of warfarin enantiomers. It has been reported that out of all the chiral selectors used, sulfated CDs resulted in the best chiral separation of racemates, yielding high resolution values of 5 or greater [22,24].

In this paper, two uncharged CD derivatives, namely Me-β-CD and 2-hydroxypropyl-β-CD (2-HP-β-CD) were first evaluated as chiral selectors for separation of warfarin enantiomers. In addition, the charged CD derivative, HS-β-CD, was also evaluated as a potential chiral selector to achieve good enantiomeric separation. This study aims to develop a chiral separation method of warfarin enantiomers using direct UV absorbance detection that is optimized in terms of the type of CD as chiral selector, CD concentration, pH of BGE buffer, BGE buffer concentration and capillary dimensions, in order to provide excellent chiral separation (resolution of greater than 5) within a short analysis time, and that is able to be applied as a rapid analytical assay for the deter-

mination of warfarin enantiomers in human plasma samples after an appropriate sample preparation step. Besides achieving improved resolution with HS-β-CD, this study also aims to improve detection sensitivity of the chiral separation method by employing a capillary of larger internal diameter (75 or 100 μm ID) instead of a 50 μm ID capillary that was used earlier by Chen and Evangelista [22].

In the previous report [22], HS-CDs were demonstrated to exhibit high resolving power with a broad range of structurally diverse racemic compounds, including warfarin that was shown to be well-resolved ($R_s = 6.1$) with HS-β-CD. Although the potential of using HS-CDs in the quantitation of enantiomers was suggested by Chen and Evangelista [22], the study was not a quantitative study but mainly a qualitative chiral CE analysis showing the general use of HS-CDs as effective chiral reagents. In addition, no other studies have yet been carried out to establish a method using HS-β-CD for the quantitation of warfarin enantiomers. Hence, the present study proposes to develop a quantitative stereospecific assay of warfarin enantiomers, using HS-β-CD as the chiral selector. This paper reports on the optimization conditions for the separation of warfarin enantiomers and the application of the developed assay method to the quantitative analysis of warfarin enantiomers in human serum samples. The final optimized assay should be validated for linearity at least in the therapeutic racemic serum warfarin concentration range of 0.5–3.0 μg/ml (or mg/l) [3] so that it can be applied for practical use.

2. Experimental

2.1. Chemicals and reagents

Racemic warfarin was obtained as the sodium salt from Sigma Chemical Co. (St. Louis, MO, USA). The internal standard used in this study, 4-hydroxycoumarin, was obtained from Fluka Chemie (Buchs, Switzerland).

The optically pure warfarin enantiomers were prepared from racemic warfarin according to the reported method (West et al., 1961) [25].

Me-β-CD and 2-HP-β-CD (degree of substitution of 0.6) were purchased from Fluka, while 20% (w/v) HS-β-CD (average degree of substitution of 12) aqueous solution together with phosphate buffer, pH 2.5 (50 mM triethylammonium phosphate) were purchased from Beckman Coulter Inc (Fullerton, CA, USA).

HPLC-grade triethylamine was obtained from J.T. Baker Co (Phillipsburg, NJ, USA). Sodium hydroxide and disodium hydrogen phosphate were from Merck (Germany). HPLC-grade acetonitrile, HPLC-grade methyl alcohol and AR-grade methyl alcohol anhydrous were obtained from Mallinkrodt (Paris). Diethyl ether of HPLC-grade was from Lab-Scan Analytical Sciences Asia Ltd (Thailand).

Human serum albumin 4.5% solution, used as substitute for human serum, was purchased from Bio Products Laboratory (UK). This commercial solution was prepared from human venous plasma of healthy voluntary donors.

All solutions were prepared using 18 MΩ (Milli-Q) water generated by a Milli-Q RG Millipore Water Purification System (Millipore, S.A., Molsheim, France).

2.2. Instrumentation

The experiments were performed on a Beckman P/ACE system 5500 (Beckman Instruments, Fullerton, CA, USA) equipped with a diode array detector capable of covering the wavelength range from 190 to 670 nm. Instrument control, data collection and data handling were carried out using Beckman P/ACE Station software system version 1.1 on a Hewlett–Packard Brio series computer (Hewlett–Packard, Singapore). Beckman CE Expert software version 1.0 was also used to calculate fluid delivery under various conditions.

The electrophoretic separations were performed using untreated fused-silica capillaries (purchased from Beckman Instruments) of 75 or 100 μm ID, with total length ranging from 27 to 57 cm (effective length to the detector ranging from 20 to 50 cm). These capillaries were assembled into a Beckman P/ACE System eCAP™ Capillary Cartridge with 100 × 800 μm aperture for UV detection.

The pH values of the background electrolytes prepared were measured using a Beckman Φ™ 110 ISFET pH meter (Beckman Instruments).

2.3. Data analysis

Resolution (R_s) was calculated by Beckman P/ACE Station software system version 1.1 based on the USP method: $R_s = 2(t_2 - t_1)/(w_1 + w_2)$, where t_1 and t_2 are apparent migration times of enantiomers 1 and 2, respectively, and w_1 and w_2 are the corresponding widths at the base of the peaks.

Statistical analysis was carried out using the one-way analysis of variance (ANOVA), with the Fisher's least significant difference (LSD) test as the post hoc multiple comparison method, from the SAS system software version 8 to determine if the differences in the migration times of the analytes in different BGE buffer concentrations were statistically significant. A P-value of less than 0.05 ($\alpha = 0.05$) was adopted as statistically significant.

2.4. Buffer and standard sample preparation

The phosphate buffer used for separation with the derivitized neutral CDs (namely Me-β-CD and 2-HP-β-CD) was prepared as a 200 mM stock solution using disodium hydrogen phosphate. Fresh 100 mM phosphate run buffers containing 8 mM of the required uncharged CD were prepared daily from the stock solution and adjusted to the desired pH using 0.1 M NaOH or 3 M HCl.

The phosphate buffer, pH 2.5 (50 mM triethylammonium phosphate) and 20% (w/v) HS-β-CD aqueous solution were diluted to prepare run buffers containing desired concentrations of triethylammonium phosphate (1, 5 or 25 mM) and HS-β-CD (2, 3, 4 or 5%). These buffers were adjusted to the desired pH with triethylamine.

Stock aqueous solution of racemic warfarin (sodium salt) was prepared at a concentration of 2 mM (i.e. 617 mg/l). Aqueous solution of 4-hydroxycoumarin (internal standard) was prepared by first dissolving 4-hydroxycoumarin with a minimal volume of 5 M NaOH and then topped up to volume to prepare a 2 mM (i.e. 324 mg/l) stock solution. Suitable dilutions were made to prepare samples of desired concentrations from these stock solutions.

Appropriate dilutions of all solutions were made with Milli-Q water. All these solutions were filtered through 0.45 μm membranes purchased from Millipore Corporation (Bedford, MA, USA) and degassed using ultrasonic bath before use.

2.5. Serum sample preparation after optimization of conditions

A 1000 mg/l stock aqueous solution of racemic warfarin (sodium salt) was prepared and diluted daily to prepare aqueous racemic drug solutions of concentrations ranging from 2 to 500 mg/l. A 50 mg/l stock aqueous solution of 4-hydroxycoumarin was prepared and diluted daily to prepare a 20 mg/l aqueous internal standard solution.

Two serum sample pre-treatment methods were investigated; the first is a serum deproteinization method while the second is a solvent extraction method. In both methods, 100 μl of human serum albumin 4.5% solution was first spiked with 10 μl of aqueous racemic warfarin solution (2, 3, 5, 20, 50, 250 or 500 mg/l) to obtain the desired albumin drug concentrations (namely 0.2, 0.3, 0.5, 2, 5, 25 or 50 mg/l, respectively), and then spiked with 10 μl of aqueous 4-hydroxycoumarin solution (20 mg/l) to obtain an internal standard concentration of 2 mg/l in the albumin sample.

In the first method, serum was deproteinized by adding 150 μl of acetonitrile to 100 μl of the above spiked albumin sample and vortex-mixed vigorously for 1 min. The mixture was then centrifuged at 3000 rpm for 15 min using a Sigma Laboratory Centrifuge (West Germany). The supernatant was injected for electrophoresis.

In the second method, the following solvent extraction procedure was performed. To culture tubes (16 × 100 mm) each containing 100 μl of spiked albumin sample, 100 μl of 3 M HCl was added to acidify it. The acidified samples were then extracted with 4 ml of diethyl ether each by shaking these capped tubes vigorously for 20 min using a rotary shaker (Gerhardt, Germany), followed by centrifugation at 2000 rpm for 15 min. The ether layers were transferred to new short-

conical bottom culture tubes, each with an anti-bumping granule added, and the ether was evaporated to dryness on a heating block at 45 °C under a stream of nitrogen. The inside of the tubes were each rinsed three times with 100 μl of methanol, with complete evaporation of the methanol after each rinse. The residue in each tube was reconstituted using 50 μl of solvent (methanol, acetonitrile or 0.05 M NaOH) and injected for electrophoresis.

2.6. Capillary electrophoresis conditions

A new capillary was conditioned by flushing with 0.1 M NaOH for 30 min and soaking overnight filled with 0.1 M NaOH, then rinsed with Milli-Q water for 30 min the following day. At the start of each day and between two consecutive injections, the capillary was rinsed under 20 psi of pressure with 0.1 M NaOH for 1 min, Milli-Q water for 2 min, the separation buffer without chiral selector for 1.8 min and the separation buffer with chiral selector for 0.2 min consecutively. The temperature of the system was set at 18 °C, unless otherwise specified. Each sample injection was introduced hydrodynamically under 0.5 psi of pressure for 6 s. Separations were performed under constant voltage using the normal polarity mode when uncharged CDs (Me-β-CD and 2-HP-β-CD) were used, and using the reverse polarity mode when HS-β-CD was used. The UV detection wavelength was set at 200 nm to provide greatest sensitivity as the spectra for warfarin and 4-hydroxycoumarin showed greatest absorbance at this wavelength.

3. Results and discussion

3.1. Optimization of separation conditions

In order to optimize the separation conditions for chiral separation of warfarin enantiomers, the effects of the following parameters were studied: type of CD, CD concentration, pH of BGE buffer, BGE buffer concentration, capillary dimensions and duration of sample injection.

3.1.1. Effect of CD type

Three types of CDs, namely Me-β-CD, 2-HP-β-CD, and HS-β-CD, were studied to evaluate the effects of these chiral selectors on the resolution of warfarin enantiomers.

For the uncharged CDs (Me-β-CD and 2-HP-β-CD), the phosphate buffers prepared were of considerably high pH (pH > 5) so that electroosmotic flow (EOF) was maximized for the complexes of these neutral CDs with warfarin enantiomers to migrate towards the cathodic end of the capillary under the normal polarity mode. Phosphate buffers at two different pH were studied, namely pH 5.5 at which warfarin ($pK_a = 5$) is 76% ionized and pH 7.0 at which warfarin is 99% ionized. For Me-β-CD, warfarin enantiomers were almost fully separated ($R_s = 0.809$) at pH 5.5 but not at pH 7.0. These were similar to the electropherograms obtained by Gareil [17], which showed the presence of a valley between the partially resolved warfarin enantiomeric peaks ($R_s \leq 0.99$) over the pH range from 4.7 to 9.1. On the other hand, for 2-HP-β-CD, separation of warfarin enantiomers could not be obtained at pH 5.5 but partial separation was observed at pH 7.0 ($R_s = 0.242$). These results show that these two uncharged CDs are not ideal as chiral selectors for separation of warfarin enantiomers.

In contrast to the two uncharged CDs, HS-β-CD which is a poly-negatively-charged CD over the entire pH range [26] exhibits high resolving power in chiral separation of warfarin enantiomers. As HS-β-CD is negatively-charged, it moves in the opposite direction of the EOF. The triethylammonium phosphate buffer of pH 2.5 was thus used with HS-β-CD as the EOF was minimized at such a low pH [22] and hence able to facilitate a more efficient migration of the HS-β-CD towards the anode which was the detection end of the capillary under the reversed polarity mode. Moreover, since HS-β-CD is negatively-charged, it will repel molecules of similar charge and thus, a low pH buffer has to be used to ensure that warfarin (a weak acid of $pK_a = 5$) is present mainly in the uncharged form rather than in the negatively-charged form so as to allow for the complexation of uncharged warfarin enantiomers with negatively-charged HS-β-

CD. Hence, the buffer of pH 2.5 is appropriate as warfarin is 99.7% unionized at this pH.

The resolution of warfarin enantiomers using HS-β-CD as the chiral selector ($R_s = 11.610$) was markedly improved as compared with both Me-β-CD and 2-HP-β-CD. However, the migration times for the warfarin enantiomers were longer when HS-β-CD was used. Nevertheless, among these three derivitized CDs, HS-β-CD provides the best enantiomeric separation for warfarin.

3.1.2. Effect of HS-β-CD concentration

The effect of the concentration of HS-β-CD was investigated by varying it from 2 to 5% in order to determine an optimum concentration at which migration times for warfarin enantiomers could be shortened with no loss in resolution. As illustrated in Fig. 1, the analysis time was significantly shortened and resolution of warfarin enantiomers was improved as the concentration of HS-β-CD increased. An increased HS-β-CD concentration increases the probability of complexation with warfarin enantiomers and also increases solute migration once complexation occurs. Thus, 5% HS-β-CD is considered optimal as it provides the fastest analysis time with best resolution.

3.1.3. Effect of pH of BGE buffer

The role of the pH of the BGE buffer where chiral separation takes place is important as it affects EOF which has a strong influence on the mobility and hence the migration time of HS-β-CD. Moreover, pH has a direct effect on the ionization of warfarin and hence its interaction with HS-β-CD. Consequently, the electrophoretic mobility and enantioselectivity of warfarin enantiomers are affected. Therefore, pH variation in the range from 2.5 to 7.5 was studied to determine the optimal pH, using the optimum concentration of 5% HS-β-CD as the chiral selector.

An increase in pH of the BGE buffer was found to result in an expected increase in the migration time of the analytes as shown in Fig. 2. This is due to the fact that as pH increases, EOF is maximized and this results in a strong opposing effect against HS-β-CD which migrates in the opposite direction to the EOF. In addition, the percentage of warfarin being ionized (negatively-charged) increases from 0.3 to 99% as pH increases from 2.5 to 7.5, leading to a decrease in interaction between warfarin enantiomers and HS-β-CD. This decreases the probability of complexation in occurring, thus increasing migration time of warfarin enantiomers. It was also found that the migration time precision for repeated injections (Table 1) at pH 2.5 is good but worsened from pH 5.5 to 7.5. This was most probably due to the alteration of buffer pH in a secondary manner by ion depletion of the buffer caused by repetitive use of the same separation buffers [27]. A different vial containing fresh buffer was used solely to refill the capillary before every separation so that the pH within the capillary would definitely be at the desired pH. The two separation buffers were used only for separation and pH alterations occurred in these two buffers after every run. Changes in the pH of the inlet separation buffer and those of the outlet separation vial were observed after each of three consecutive runs. This secondary alteration of pH appeared to affect the precision for buffers at higher pH to a more severe extent. The results thus demonstrate that the triethylammonium phosphate buffer at pH 2.5 is the pH that is most optimal for successful chiral separation within the shortest time with the best precision for repeated consecutive injections.

3.1.4. Effect of BGE buffer concentration

After optimizing the pH of the BGE buffer, another series of experiments were performed at the optimal pH of 2.5 with 5% HS-β-CD to determine the optimum BGE buffer concentration. Three concentrations, namely 1, 5 and 25 mM, were studied. The average migration times of the analytes in 1 mM buffer (9.272, 19.654 and 23.708 min for internal standard, *R*-warfarin and *S*-warfarin, respectively) and 5 mM buffer (9.372, 19.590 and 23.447 min for internal standard, *R*-warfarin and *S*-warfarin, respectively) were both similarly faster than those in 25 mM buffer (10.110, 22.576 and 28.170 min for internal standard, *R*-warfarin and *S*-warfarin, respectively) (Fig. 3A). There were significant differences in migration time among the three buffer concentrations for the internal standard ($P = 0.0163$), *R*-

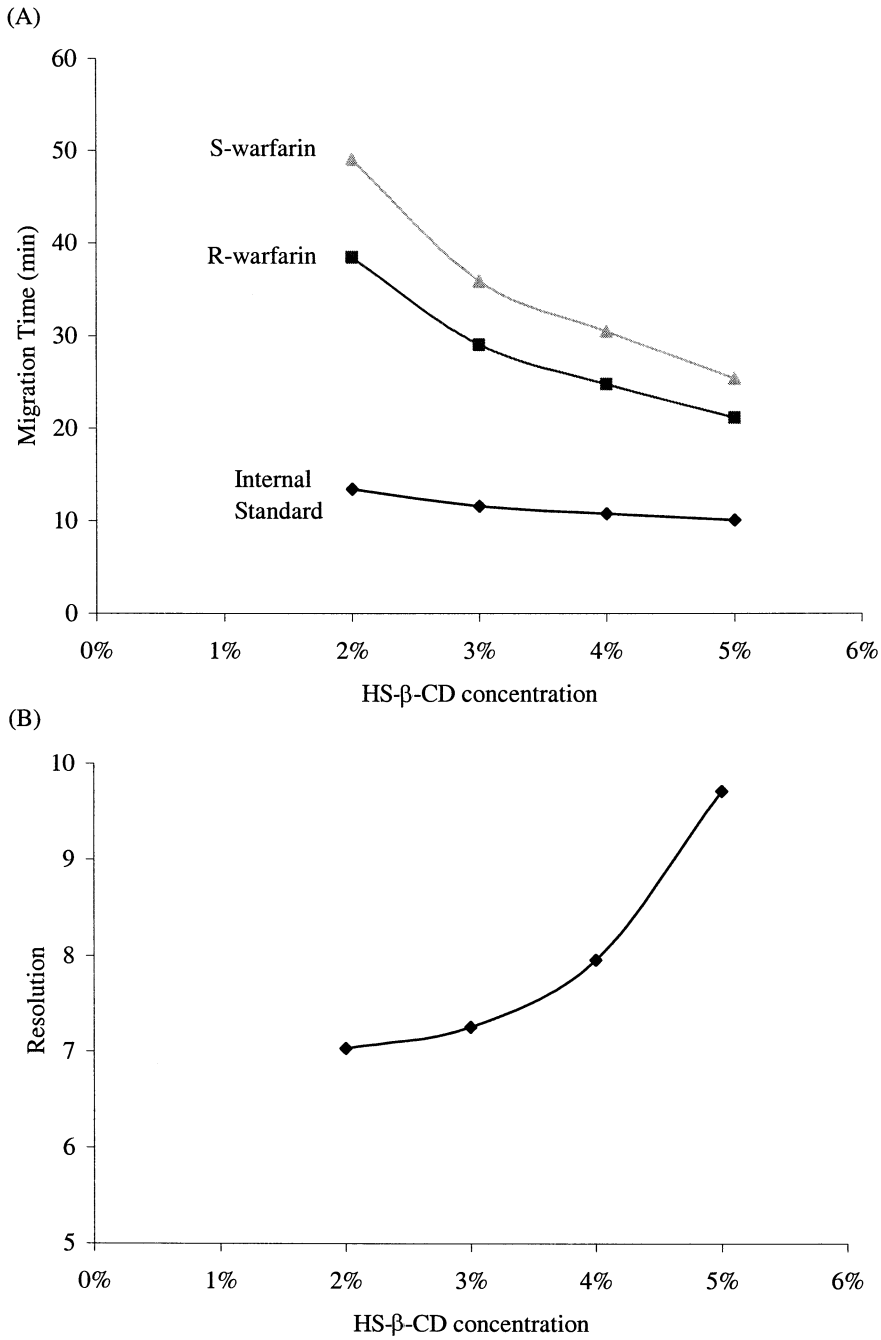

Fig. 1. Effect of HS-β-CD concentration on the, (A) migration times of the analytes, and (B) resolution of warfarin enantiomers. Conditions, Fused silica capillary, 75 μm ID × 37 cm total length (effective length 30 cm); applied voltage, 9 kV; running current, 100–150 μA; Reverse polarity; temperature, 18 °C; Hydrodynamic injection time, 6 s; UV detection wavelength, 200 nm; Background electrolyte, phosphate buffer pH 2.5 (25 mM triethylammonium phosphate); Chiral selector, HS-β-CD at the following concentrations; 2, 3, 4 and 5%; 0.1 mM internal standard (4-hydroxycoumarin); 0.1 mM racemic warfarin.

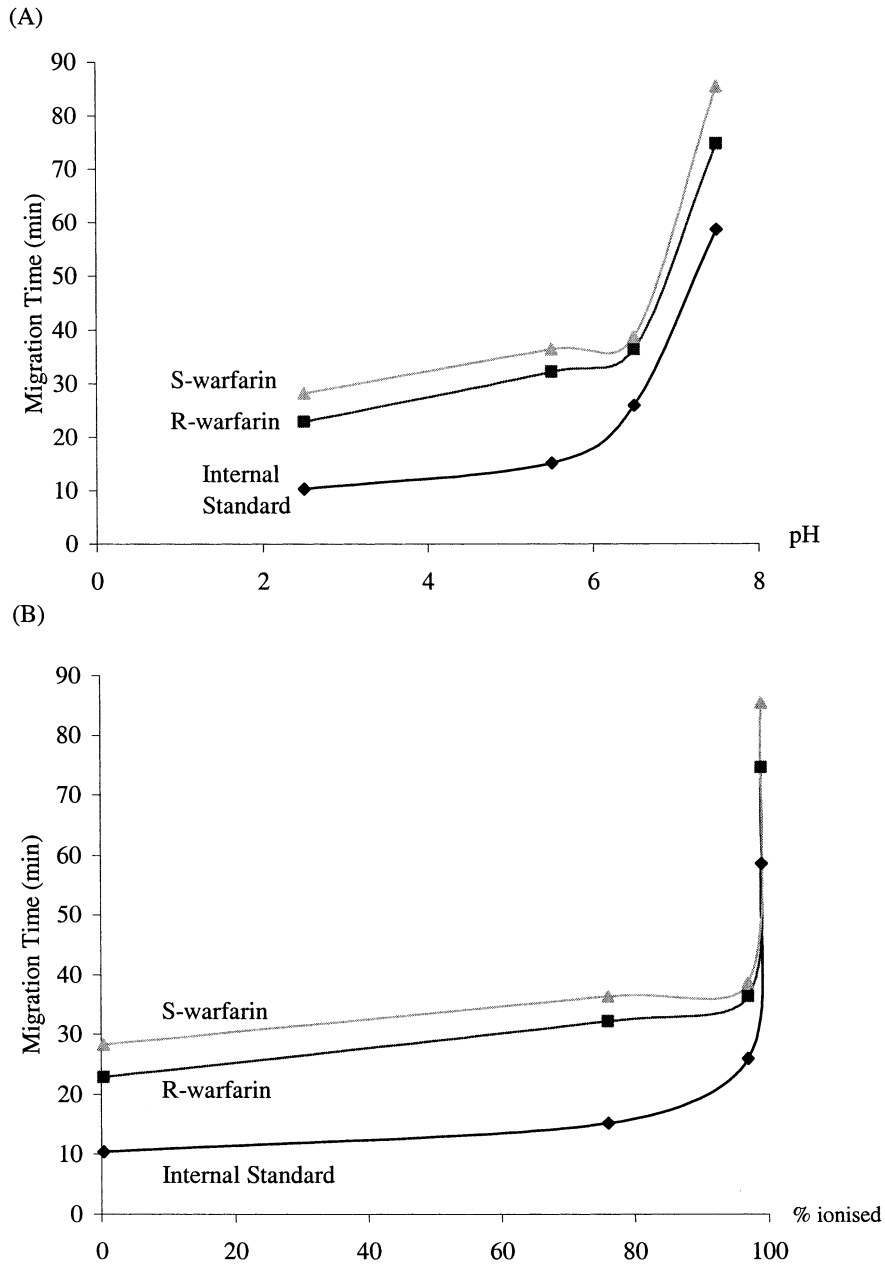

Fig. 2. Effect of (A) pH and (B)% of warfarin ionized, on migration times of analytes. Conditions; fused silica capillary, 75 μm ID × 37 cm total length (effective length 30 cm); applied voltage, 9 kV; running current, 150–180 μA; reverse polarity; temperature, 18 °C; Hydrodynamic injection time, 6 s; UV detection wavelength, 200 nm; background electrolyte, phosphate buffer (25 mM triethylammonium phosphate); chiral selector, HS-β-CD 5%; 0.1 mM internal standard (4-hydroxycoumarin); 0.1 mM racemic warfarin.

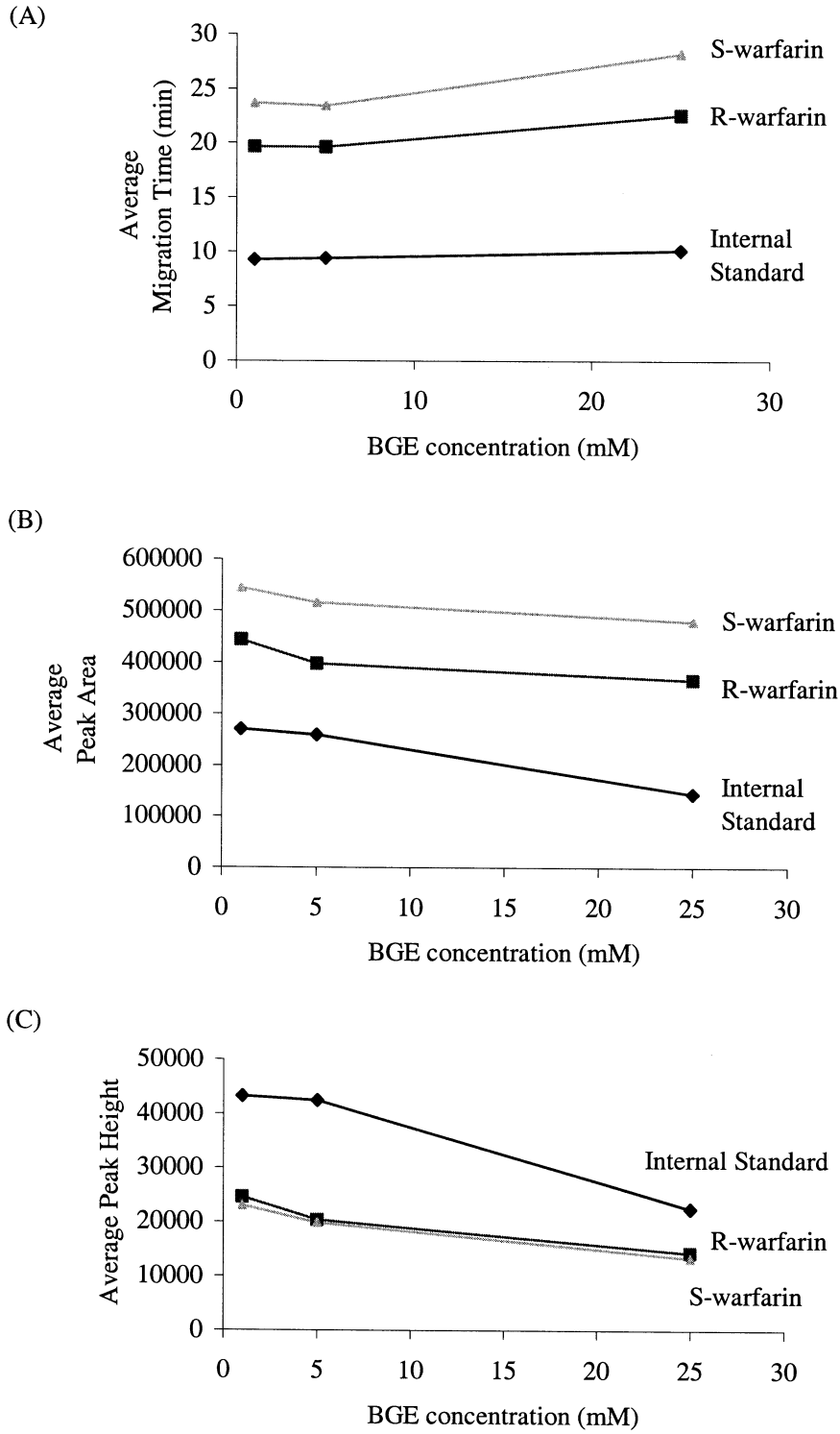
(A)
Average Migration Time (min)
30
25
20
15
10
5
0
0
10
20
30
BGE concentration (mM)
S-warfarin
R-warfarin
Internal Standard
(B)
Average Peak Area
600000
500000
400000
300000
200000
100000
0
0
5
10
15
20
25
30
BGE concentration (mM)
S-warfarin
R-warfarin
Internal Standard
(C)
Average Peak Height
50000
40000
30000
20000
10000
0
0
5
10
15
20
25
30
BGE concentration (mM)
Internal Standard
R-warfarin
S-warfarin

Fig. 3.

Table 1
Migration time precision for repeated injections ($n = 3$) at different background electrolyte (BGE) buffer pH

pH	% of warfarin ionised		Migration time for		
			Internal standard	*R*-warfarin	*S*-warfarin
2.5	0.3152	Average (min)	10.110	22.576	28.170
		S.D.	0.223	0.413	0.716
		R.S.D. (%):	2.20	1.83	2.54
5.5	75.9747	Average (min)	12.905	28.184	33.430
		S.D.	1.953	3.489	2.599
		R.S.D. (%)	15.13	12.38	7.77
6.5	96.9347	Average (min)	17.345	31.424	36.284
		S.D.	7.490	4.492	2.229
		R.S.D. (%)	43.18	14.30	6.14
7.5	99.0099	Average (min)	29.743	41.437	46.978
		S.D.	25.020	29.492	33.658
		R.S.D. (%):	84.12	71.17	71.65

Conditions; fused silica capillary, 75 μm ID × 37 cm total length (effective length 30 cm); applied voltage, 9 kV; running current, 150–180 μA; reverse polarity; temperature, 18 °C; hydrodynamic injection time, 6 s; UV detection wavelength, 200 nm; background electrolyte, phosphate buffer (25 mM triethylammonium phosphate); chiral selector, HS-β-CD 5%; 0.1 mM internal standard (4-hydroxycoumarin); 0.1 mM racemic warfarin.

warfarin ($P = 0.0013$) and *S*-warfarin ($P = 0.0003$). The Fisher's LSD test in ANOVA further indicated that the migration times of the analytesanalytes in 25 mM buffer were significantly different from those in both the 1 and 5 mM buffers while there was no significant difference between the migration times of the analytes in the 1 and 5 mM buffers. Sensitivity was the best for 1 mM buffer as the average peak areas (Fig. 3B) and average peak heights (Fig. 3C) for the analytes were the largest. In addition, 1 mM buffer also resulted in the best resolution of warfarin enantiomers ($R_s = 7.341$ for 1 mM buffer; $R_s = 6.978$ for 5 mM buffer; $R_s = 5.828$ for 25 mM buffer). Hence, 1 mM was considered to be the optimum buffer concentration. Moreover, the lower the concentration of the buffer, the lower would be the current during separation [27]. Since the ionic strength of the poly-negatively-charged HS-β-CD is high in the buffer resulting in a high current during separation, it would be ideal to use a low concentration buffer of 1 mM as a compromise to maintain acceptable current level and to prevent excessive Joule heating.

3.1.5. Effect of capillary dimensions

In an attempt to enhance detection sensitivity and shorten analysis time, the effect of changes in the capillary length and internal diameter were studied. By increasing the internal diameter, hydrodynamic injection for the same duration results in an increase in the injected sample volume, thus improved sensitivity is expected. Two capillaries, each with a total length of 37 cm and having an internal diameter of 75 and 100 μm, respectively, were compared. For both capillaries, the same injection duration of 6 s was employed and the calculated sample volumes injected were 41.16 and 130 nl for the capillaries with 75 and 100 μm ID, respectively. However, sensitivity was not improved for the capillary with 100 μm ID due to the smaller peak areas and peak heights obtained as compared with those of the 75 μm ID. This was

Fig. 3. Effect of BGE buffer concentration on the (A) average migration times, (B) average peak areas and (C) average peak heights of analytes. Conditions; fused silica capillary, 75 μm ID × 37 cm total length (effective length 30 cm); applied voltage, 9 kV; running current, 150–180 μA; Reverse polarity; temperature, 18 °C; hydrodynamic injection time, 6 s; UV detection wavelength, 200 nm; background electrolyte, phosphate buffer pH 2.5 (triethylammonium phosphate) at concentrations of 1, 5 and 25 mM; chiral selector, 5% HS-β-CD; 0.1 mM internal standard (4-hydroxycoumarin); 0.1 mM racemic warfarin.

because a lower voltage had to be applied for the 100 μm ID capillary as an increased internal diameter leads to a decrease in the surface-to-volume ratio, causing increased Joule heating and increased current during separation. Thus, the 100 μm ID capillary could only attain a maximum voltage of 7 kV at maximum current of 250 μA whereas separation could be carried out at a maximum voltage of 9 kV for the 75 μm ID capillary. As a result of the lower voltage, the migration times of the warfarin enantiomers were also slower for the 100 μm ID capillary. Due to these disadvantages of increasing the internal diameter, the capillary with 75 μm ID was considered more favorable.

The high resolving power of HS-β-CD allows for the use of short capillaries to reduce analysis time significantly without any loss in resolution. However, as capillary length decreases, a concomitant increase in current occurs and hence, a lower voltage has to be applied instead. Nevertheless, migration times for warfarin enantiomers were still significantly shortened when capillary length decreased from a total length of 57 cm (total migration time: 26 min) to 27 cm (total migration time: 11 min) even though applied voltage had to be decreased.

Thus, a capillary with dimensions of 27 cm total length and 75 μm ID was considered optimal for efficient and effective chiral separation of warfarin enantiomers.

3.1.6. Effect of increasing duration of sample injection

A longer injection duration of 12 s was tried to improve sensitivity but this resulted in distorted peak shapes due to overloading of the capillary. It is generally recommended that the maximum volume of sample injected be limited to 5% of the total capillary volume [27]; hence, the maximum injection duration for the capillary with optimal dimensions of 27 cm × 75 μm ID (capillary volume of 1193 nl) is 6 s (sample volume of 56.4 nl). This injection duration was thus used.

3.2. Optimal conditions and assay validation

The fully optimized operating conditions for the chiral separation of warfarin enantiomers are shown in Fig. 4. Under the reversed polarity mode, the migration order of warfarin enantiomers, as determined by the analyses using the respective pure enantiomeric forms of warfarin, is *R*-warfarin followed by *S*-warfarin.

Under the fully optimized conditions using HS-β-CD as the chiral selector, the warfarin enantiomers were well-separated with a high resolution similar to that reported by Chen and Evangelista ($R_s = 6.1$) [22]. However, the electrophoretic migration time taken (11 min) was longer than that reported in the earlier published paper (6 min) [22]. This was due to the use of a capillary with a larger internal diameter (75 μm ID), with the aim of improving detection sensitivity. As a result of the increase in internal diameter, a lower voltage (7 kV) had to be applied to prevent excessive Joule heating, hence leading to a longer electrophoretic migration time. Nevertheless, detection sensitivity could be improved by using a capillary of larger internal diameter (75 μm ID) as compared with that in the earlier publication (50 μm ID) [22] since an increase in the internal diameter results in an increase in the sample volume injected hydrodynamically for the same duration.

The warfarin enantiomer concentration and average relative peak area (warfarin enantiomer to internal standard peak area ratio) for seven aqueous warfarin standards (each containing the internal standard at 2 mg/l) correlated linearly in the concentration range from 0.1 to 25 mg/l ($y = 0.56390x + 0.06114$, $r^2 = 0.99973$ for *R*-warfarin and $y = 0.68205x + 0.05212$, $r^2 = 0.99995$ for *S*-warfarin). For aqueous warfarin standards, the limit of detection (LOD) and limit of quantitation (LOQ) in terms of warfarin enantiomer concentrations were 0.1 mg/l (3 × baseline noise) and 0.3 mg/l (10 × baseline noise), respectively, for *R*-warfarin while those for *S*-warfarin were 0.1 mg/l (3 × baseline noise) and 0.25 mg/l (10 × baseline noise), respectively.

The repeatability (intra-day precision) of migration time and relative peak area for aqueous warfarin standards was evaluated using three independently-prepared samples each ($n = 3$) for seven different warfarin enantiomer concentrations ranging from 0.1 to 25 mg/l, with the internal standard

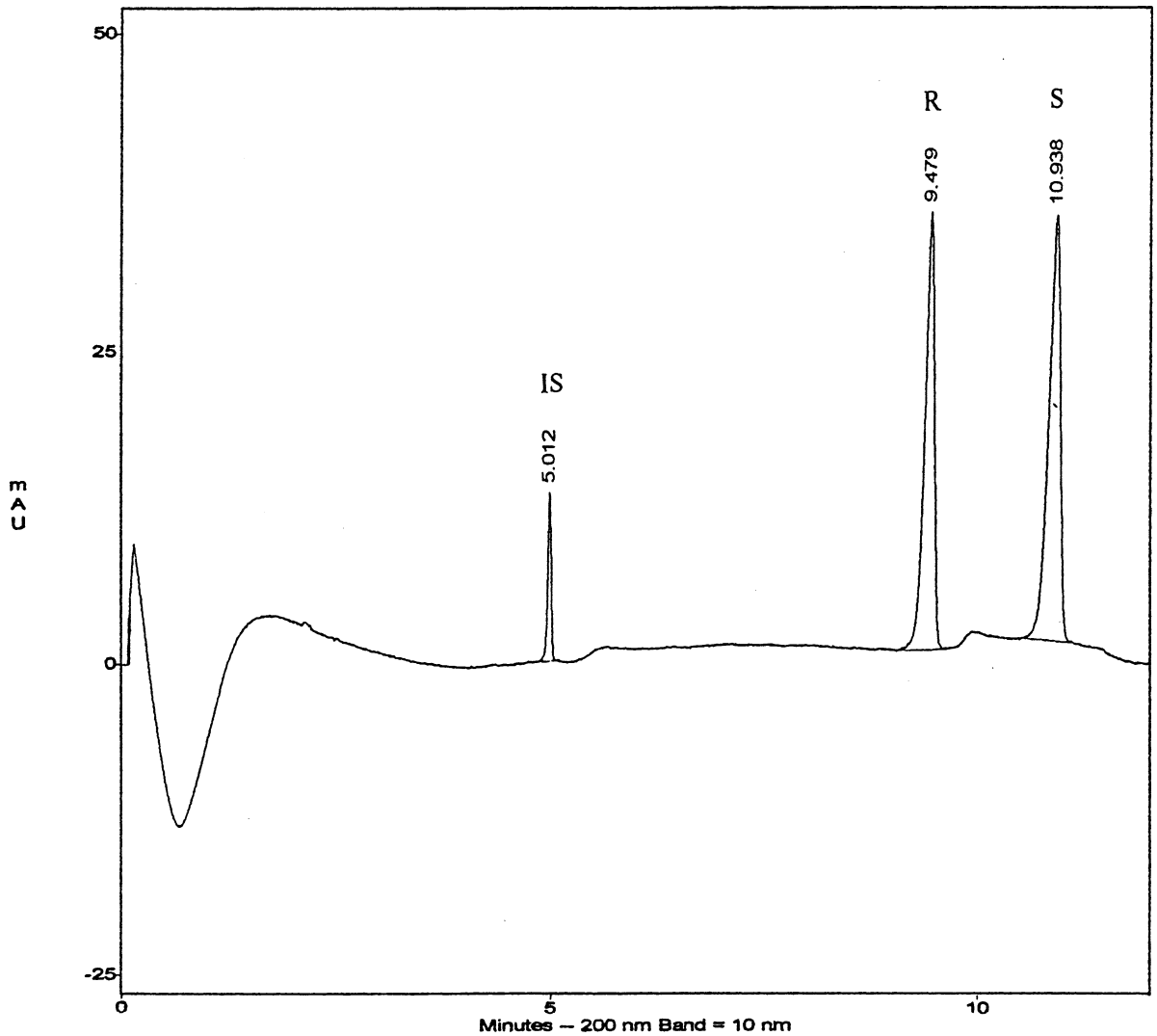

Fig. 4. Electropherogram representing chiral separation of racemic warfarin under optimized conditions. Conditions; fused silica capillary, 75 μm ID × 27 cm total length (effective length 20 cm); applied voltage, 7 kV; running current, 153 μA; reverse polarity; temperature, 18 °C; hydrodynamic injection time, 6 s; UV detection wavelength, 200 nm; background electrolyte, phosphate buffer pH 2.5 (1 mM triethylammonium phosphate); Chiral selector, 5% HS-β-CD; 2 mg/l internal standard (4-hydroxycoumarin); 25 mg/l racemic warfarin. Peaks, IS = internal standard; R = *R*-warfarin; S = *S*-warfarin.

(4-hydroxycoumarin) concentration fixed at 2 mg/l. The intermediate precision (inter day precision) study was conducted over 3 consecutive days. The mean, standard deviation (S.D.) and relative standard deviation (R.S.D.) values were calculated for migration time and relative peak area. The R.S.D. values for migration time and relative peak area were less than 5.5 and 9.2%, respectively, for intra-day precision, and less than 4.4 and 5.1%, respectively, for inter-day precision.

3.3. Application of optimized conditions for the analysis of warfarin enantiomers in serum albumin samples

Prior to the determination of warfarin enan-

tiomers in serum samples using the optimized CE conditions obtained, a serum sample pre-treatment step had to be employed. The solvent extraction method was a suitable sample preparation method for warfarin enantiomers in human serum [5,17] but it was quite time-consuming and tedious. A fast and simple technique of serum deproteinization as a sample preparation method has been developed [27] and reported to be adequate for sample clean-up of serum with valproic acid [28], and serum, urine and cerebrospinal fluids with xanthine [29]. This method of using acetonitrile to remove serum proteins was attempted to study the feasibility of using it as a simple and rapid method for preparing biological fluids for analysis using CE.

3.3.1. Serum deproteinization

It has been reported that the ratio of serum to acetonitrile of 2:3 is adequate for removing serum proteins especially albumin [27,28]. However, an attempt using this sample clean-up method on human serum albumin solution spiked with warfarin was unsuccessful because of the presence of interfering peaks and noise in the background of the electropherograms. Moreover, as over 99% of warfarin is bound to plasma albumin [1], most of the warfarin in the human serum albumin solution would have been removed together with the serum proteins during serum deproteinization, resulting in warfarin peaks being too small to be detectable and differentiable from background noise. This was also partly due to the sample being diluted by the addition of acetonitrile to serum albumin. Thus, serum deproteinization method was not appropriate for serum sample preparation for warfarin analysis and the conventional extraction method had to be used instead.

3.3.2. Solvent extraction

The extraction procedure was carried out as the serum sample pre-treatment method since serum sample preparation by solvent extraction had been employed successfully for determination of warfarin enantiomers in plasma samples by CE [5,17]. The reconstitution of the residue after extraction was tried out using methanol, acetonitrile and 0.05 M NaOH. Methanol was chosen as the ideal reconstitution solvent as it provided the least interference and background noise. Besides, the warfarin peaks were more well-defined as compared with reconstitution with acetonitrile or 0.05 M NaOH.

After solvent extraction and reconstitution with methanol, the determination of warfarin enantiomers in spiked serum albumin samples was performed using the optimized conditions. It was first ascertained that a blank serum albumin sample gave no interfering peaks within the range of migration times of warfarin enantiomers and the internal standard (Fig. 5).

To assess the recovery of warfarin enantiomers from serum by this pre-treatment procedure, the racemic drug was added to drug-free serum and assayed with the internal standard (2 mg/l). The absolute recoveries of warfarin enantiomers were examined at three different warfarin enantiomer concentrations of 0.15, 0.25 and 2.5 mg/l, each assayed in triplicates. The average extraction recoveries of internal standard, *R*-warfarin and *S*-warfarin were within 14.0–19.1% (R.S.D. $\leq$ 9.77%), 65.3–79.8% (R.S.D. $\leq$ 10%) and 52.7–66.2% (R.S.D. $\leq$ 9.82%), respectively.

A study of repeatability (intra-day precision) ($n = 3$) and intermediate precision (inter-day precision) (over 3 consecutive days) with serum albumin samples spiked with racemic warfarin at enantiomer concentrations of 0.15, 0.25 and 2.5 mg/l was conducted. The R.S.D. values for migration time and relative peak area were less than 6.2 and 9.4%, respectively, for intra-day precision, and less than 9.6% for both migration time and relative peak area for inter-day precision.

Although the recovery of the internal standard was low (14.0–19.1%), the intra-day and inter-day precision of migration time and relative peak area were within acceptable limits. Hence, the use of this solvent extraction method as the serum sample pre-treatment procedure for warfarin analysis is feasible.

Linearity was evaluated in the warfarin enantiomer concentration range from 0.1 to 25 mg/l for seven spiked serum albumin samples (each being spiked with 2 mg/l internal standard). A high degree of correlation was found between

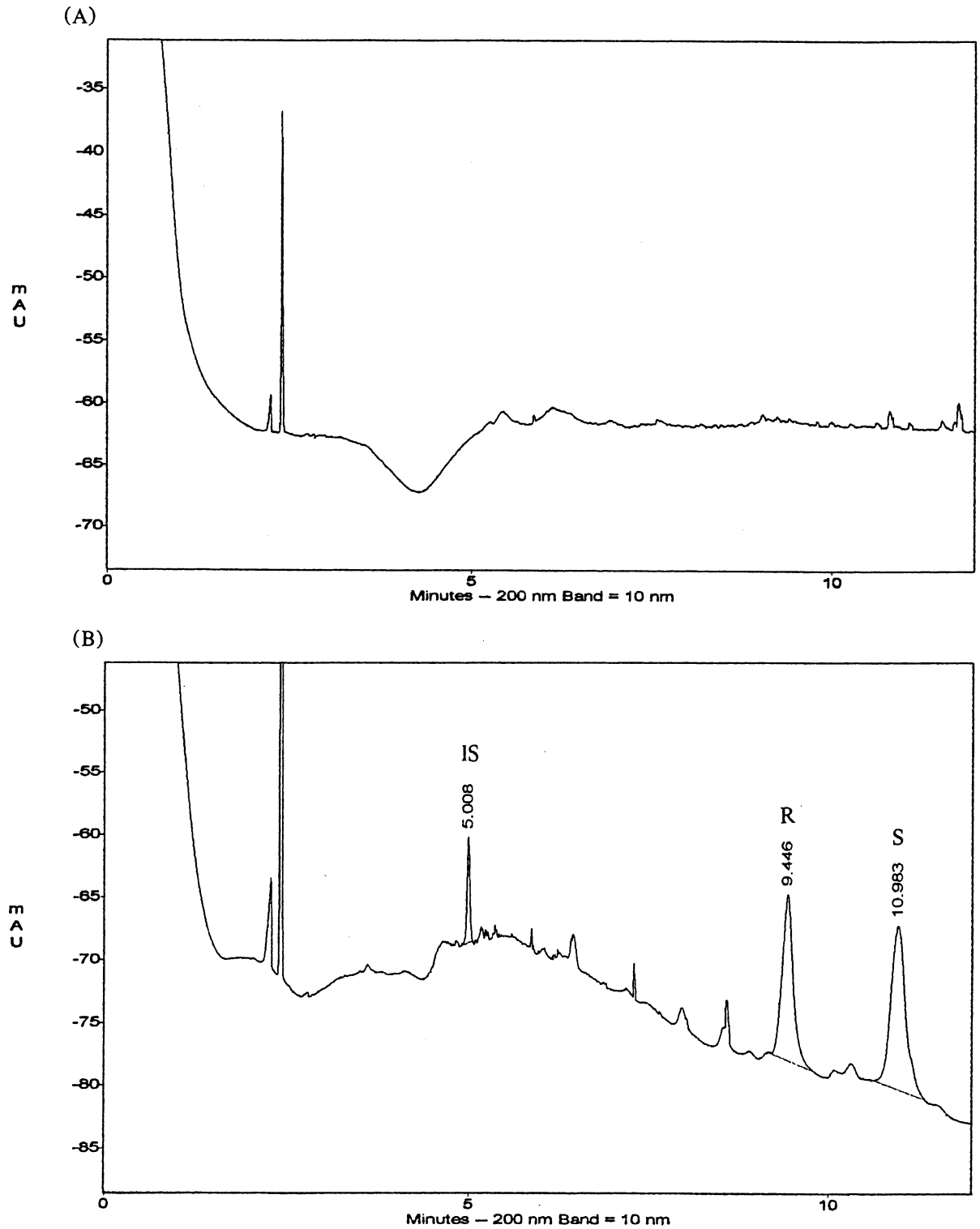
(A)
mAU
-35
-40
-45
-50
-55
-60
-65
-70
0
5
10
Minutes — 200 nm Band = 10 nm
(B)
mAU
-50
-55
-60
-65
-70
-75
-80
-85
IS
5.008
R
9.446
S
10.983
0
5
10
Minutes — 200 nm Band = 10 nm

Fig. 5.

Table 2
Absolute percentage (%) error of warfarin enantiomer concentration between actual and measured warfarin enantiomer concentrations in simulated serum samples to demonstrate applicability of the assay method using solvent extraction as the serum sample pre-treatment procedure for the determination of the concentrations of warfarin enantiomers in spiked human serum samples

Actual warfarin enantiomer concentration (mg/l)	Measured warfarin enantiomer concentration (mg/l) in simulated human serum samples		Absolute% error between actual and measured warfarin enantiomer concentration	
	R-warfarin	*S*-warfarin	*R*-warfarin	*S*-warfarin
0.10	0.093	0.091	7.00	9.00
0.15	0.138	0.135	8.00	10.00
0.25	0.275	0.240	10.00	4.00
1.00	1.095	1.073	9.50	7.30
2.50	2.654	2.693	6.16	7.72

warfarin enantiomer concentrations and relative peak areas, with linearity data of $y = 1.9758x + 0.2265$ ($r^2 = 0.9991$) for *R*-warfarin and $y = 2.2711x + 0.344$ ($r^2 = 0.9998$) for *S*-warfarin.

For spiked serum albumin samples, the limits of detection (LOD) and quantitation (LOQ) were evaluated as warfarin enantiomer concentrations of 0.05 mg/l (3 × baseline noise) and 0.15 mg/l (10 × baseline noise), respectively, for each warfarin enantiomer. The LOD value obtained in this study was better than that reported using Me-β-CD as the chiral selector (LOD = 0.2 mg/l for each warfarin enantiomer) [17] and that using Glucidex2® maltodextrin (LOD = 1 mg/l for each warfarin enantiomer) [5]. No comparisons could be made for the LOQ value obtained, as no LOQ value was reported in the previous studies.

Due to the much lower recovery of the internal standard (14.0–19.1%) as compared with that of both *R*-warfarin (65.3–79.8%) and *S*-warfarin (52.7–66.2%), the calculated peak area ratios were constantly much larger than those without extraction in the aqueous standards. Still, the LOD and LOQ for spiked serum samples were found to be better (two times more sensitive) than those for the aqueous standards. This is mainly due to the reconstitution of the dried extract in a smaller volume of the solvent prior to CE analysis.

The applicability of this optimized assay method with solvent extraction as the serum sample pre-treatment procedure was also assessed by testing on simulated spiked human serum samples in the warfarin enantiomer concentration range from 0.1 to 2.5 mg/l which encompasses the therapeutic racemic serum warfarin concentration range of 0.5 to 3.0 mg/l. The simulated plasma samples were assayed under blind conditions for the determination of the concentrations of warfarin enantiomers. After the code was broken, the plasma drug concentrations were subject to data analysis. Table 2 showed that the absolute percentage errors of warfarin enantiomer concentration were less than or equal to 10%.

4. Conclusions

A chiral separation method based on direct UV absorbance detection for enantioselective

Fig. 5. Representative electropherograms of (A) blank serum albumin after solvent extraction and (B) spiked human serum albumin sample (racemic warfarin, 5 mg/l; internal standard: 4-hydroxycoumarin, 2 mg/l). Conditions; fused silica capillary, 75 μm ID × 27 cm total length (effective length 20 cm); applied voltage, 7 kV; running current, 130–190 μA; reverse polarity; temperature, 18 °C; hydrodynamic injection time, 6 s; UV detection wavelength, 200 nm; background electrolyte, phosphate buffer pH 2.5 (1 mM triethylammonium phosphate); Chiral selector, 5% HS-β-CD. Peaks, IS = internal standard; R = *R*-warfarin; S = *S*-warfarin.

analysis of warfarin enantiomers using CE was developed and optimized. HS-β-CD was selected as the most effective chiral selector since it was demonstrated and also reported to have the highest resolving power [22,24] as compared with other chiral selectors, including maltodextrin [5] and Me-β-CD [17] that were used in the separation and quantitation of warfarin enantiomers in plasma samples. Although warfarin enantiomers were shown to be well-resolved by HS-β-CD in the qualitative study by Chen and Evangelista and the potential use of HS-β-CD in the quantitation of enantiomers was also suggested [22], no actual study in the quantitative analysis of warfarin enantiomers using HS-β-CD has been reported. This area, which is yet to be ventured into, was thus explored in this study and the applicability of the optimized assay method to the quantitation of warfarin enantiomers in human serum samples was demonstrated.

In this study, the assay method was first developed and optimized. A high resolution of warfarin enantiomers with electrophoresis time of around 11 min was achieved using HS-β-CD as the chiral selector. This method was validated and was shown to have acceptable intra-day and inter-day precision. The LOD and LOQ evaluated as warfarin enantiomer concentrations were 0.05 and 0.15 mg/l, respectively, for each warfarin enantiomer in spiked serum albumin samples. Linearity was demonstrated for warfarin extracted from spiked serum albumin samples in the warfarin enantiomer concentration range of 0.1–25 mg/l. Since this range encompasses the therapeutic racemic serum warfarin concentration range of 0.5–3.0 mg/l, this assay method may prove to be useful for stereoselective pharmacokinetic investigation of warfarin enantiomers in patients on warfarin therapy.

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