Journal of Chromatography, 615 (1993) 317–325
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6786

Separation and determination of warfarin enantiomers in human plasma samples by capillary zone electrophoresis using a methylated β-cyclodextrin-containing electrolyte

P. Gareil*

Laboratoire de Chimie Analytique de l'Ecole Supérieure de Physique et Chimie Industrielles de la Ville de Paris, 10 Rue Vauquelin, 75231 Paris Cedex 05 (France)

J. P. Gramond and F. Guyon

Laboratoire de Chimie Analytique de l'Université René Descartes, 4 Avenue de l'Observatoire, 75006 Paris (France)

(First received June 30th, 1992; revised manuscript received February 12th, 1993)

ABSTRACT

A methyl β-cyclodextrin with a degree of substitution of 1.8 proved to be an effective chiral selector, among other cyclodextrins tested, for the separation of warfarin enantiomers by capillary electrophoresis. The operating conditions were optimized with respect to electrolyte composition (buffer pH, ionic strength, cyclodextrin concentration, methanol content) and applied voltage. The influence of a high ionic strength on the resolution was clearly shown. A baseline separation can be obtained in less than 15 min with an efficiency of *ca.* 250 000 theoretical plates. These conditions were applied to the determination of warfarin enantiomers in the plasma of patients under warfarin therapy. The limit of detection for the whole procedure (dichloromethane extraction followed by evaporation to dryness and capillary electrophoresis) was of the order of 0.2 mg/l ($6.5 \cdot 10^{-7}$ *M*) of each enantiomer.

INTRODUCTION

Warfarin [3-(α-acetonylbenzyl)-4-hydroxycoumarin, Fig. 1] is known to have anticoagulant (antivitamin K) properties and has been used as its racemate in therapeutic treatment for several decades. However, it has been recognized that the (*S*)-(−)-isomer is about three times as active as the (*R*)-(+)-isomer [1]. It has also been established that the two enantiomers are metabolized quite differently, and that the (*S*)-(−)-enantiomer is metabolized much faster [2]. In addition, it has been shown that the (*S*)-isomer interacts with plasma proteins more strongly than the (*R*)-isomer [3]. In spite of these data, no attempt has been made so far to produce warfarin as an optically active drug.

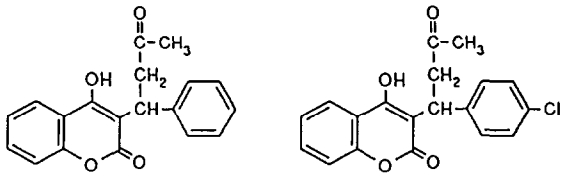

Fig. 1. Structures of warfarin (Wf) and 5-chlorowarfarin (ClWf).

Enantioselective determination methods are highly desirable to investigate further on the activity and pharmacokinetics of both optical iso-

* Corresponding author.

0378-4347/93/$06.00

mers. Column liquid chromatography with chiral stationary phases (CSPs) has been the technique of choice for this purpose. Among the CSPs reported so far are silicas coated or grafted with bovine serum albumin [4], α_1-acid glycoprotein (α_1AGP) [5], CBH1 cellulase [6] and a β-cyclodextrin (β-CD)-derivatized polymer [7]. The chiral selectivities ranged from 1.2 [6] to 1.5 [4,5] with analysis times from 10 min [5] and more than 30 min [6]. The most effective separation seems to be that achieved with the α_1AGP column, enabling determination of free warfarin enantiomers at a micromolar level in the presence of human serum albumin [5]. Recently, a sensitive stereospecific assay of warfarin in plasma including the precolumn formation of (−)-(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 and the subsequent separation with a reversed-phase column has been developed [8].

This paper reports on the development of a free solution capillary electrophoresis (FSCE) method as an alternative to the determination of warfarin enantiomers in human plasma. The anticipated advantages of this rapidly growing technique over most classical chromatographic ones are that it affords high efficiency and speed of analysis while requiring low free form amounts of the often expensive chiral selectors and very small amounts of sample. A methylated β-cyclodextrin derivative was used as the chiral selector. Previously, various cyclodextrin derivatives had already been used in FSCE for chiral separations of amino acids [9,10] and several pharmaceuticals [11–14].

EXPERIMENTAL

Apparatus

The separations were conducted with an automated, commercially available capillary electrophoresis instrument [Model 270 A from Applied Biosystems (ABI), Foster City, CA, USA] designed for on-column absorbance detection with continuous wavelength setting. The electropherograms were monitored either with a two-channel BD 40 strip chart recorder (Kipp and Zonen, Delft, Netherlands), which allowed simultaneous current intensity recording, or with a Chromjet data integrator (Spectra-Physics, San Jose, CA, USA).

Materials.

The fused-silica capillaries (72 cm × 50 μm I.D.) used throughout, also from ABI, were externally coated with polyimide. The detection cell was 50 cm from the injection end. The inner surface was bare fused silica. The electrolytes were prepared from the purest commercially available, analytical-reagent-grade products, HPLC-grade methanol, and Milli-Q-quality water (Waters-Millipore, Milford, MA, USA). Underivatized β-CD was purchased from Aldrich (Strasbourg, France) and methylated β-cyclodextrin (Me-β-CD) (degree of substitution 1.8) was a gift from Waker-Chemie (Munich, Germany). All electrolytes were degassed for 15 min in an ultrasonic bath and filtered through 0.45-μm Millipore Millex units prior to loading into the electrolyte vessels.

Capillary electrophoresis.

New capillaries were conditioned by flushing with 1 *M* sodium hydroxide and pure water for 30 min each and then equilibrating with the operating buffers for 10–30 min, according to the pH. Between two consecutive injections, the capillary was rinsed with 0.1 *M* sodium hydroxide for 1 min and the operating buffer for 5 min. Periodically, the procedure used for new capillaries was repeated to restore the state of the inner surface. Sample injections were carried out hydrodynamically, using the 16.9 kPa vacuum line delivered by the instrument. The temperature was set at 25°C. The volume injected under these conditions was *ca.* 3.5 nl per second. The voltage applied for separations was 20 kV, unless otherwise specified, with the positive electrode being located on the injection side. The optimized composition of the operating buffer is given below (see legend of Fig. 3). The detection wavelength was set at 310 nm, *i.e.* at the upper wavelength maximum of the absorbance spectrum of warfarin, to provide greater detection selectivity. The detector rise time was 1 s.

Sample preparation.

A standard mixture containing 0.65 m*M* warfarin and 0.23 m*M* 5-chlorowarfarin (Fig. 1) was prepared by allowing 5 mg of warfarin (Aldrich, molecular mass 308.3) and 2 mg of 5-chlorowarfarin (Aldrich, molecular mass 342.7) to dissolve in 25 ml of water–methanol (20:80, v/v). All samples were sonicated and filtered on 0.45-μm Millex units prior to injection into the capillary. To 1 ml of plasma of a patient under warfarin therapy (or 1 ml of warfarin-spiked blank plasma) was added 0.05 ml of a 25 mg/l solution of 5-chlorowarfarin as internal standard, followed by 0.1 ml of 1 *M* hydrochloric acid. The acidified samples were then extracted with 5 ml of dichloromethane by stirring for 10 min. After separation of the phases by centrifugation (500 *g*, 5 min), the organic layer was transferred to an evaporation tube and then evaporated at 40°C under a stream of nitrogen. The residue was redissolved in 0.05 ml of the separation electrolyte. A three-point standard curve was constructed for each set of samples by preparing veal plasmas spiked with 1, 2 and 3 mg/l warfarin.

RESULTS AND DISCUSSION.

Like many aromatic compounds, warfarin forms inclusion complexes with β-CD, a cyclic oligosaccharide molecule made of seven glucose units, of unique open cylindro-conical shape. Computer models reveal that the coumarin moiety of warfarin penetrates into the hydrophobic cyclodextrin cavity, while the phenyl substituent remains outside the larger mouth of the cone [15]. It is also well known that CDs can provide enantioselectivity if the formation constants of the inclusion complexes of a pair of enantiomers are quite different. However, to this end, several conditions should be met. Especially, it seems that the chiral centre of the molecule should be located near to the opening of the CD, so that at least one of its substituents can interact with the polar groups of the CD rim [7,13]. As this is not the case for warfarin and β-CD, no chiral resolution was expected to ocur with β-CD-CSP in chromatography, which has turned out to be true experimentally [15]. The same result also appeared to us in FSCE, as we never succeeded in obtaining even a hint of a chiral resolution, whatever the electrolyte composition, any time β-CD was added to those electrolytes.

In contrast, a partial resolution was soon observed in preliminary experiments with Me-β-CD. In order to optimize the separation conditions, the effect of the following parameters was investigated with standard warfarin racemate samples: electrolyte buffer pH and ionic strength, Me-β-CD concentration, methanol content, electroosmotic velocity and applied voltage. However, no attempt was made to optimize the capillary dimensions. As previously suggested, 5-chlorowarfarin was tested as a potential internal standard, and added to the warfarin samples.

Effect of electrolyte buffer pH

The electrolyte buffer pH was first tested in a range from 4.7 to 9.1, encompassing the value of the acidic p*K* of the warfarin phenolic function, 5.1 [16]. Sodium chloride (100 m*M*) was added to various 20 m*M* buffers, to maintain the ionic strength almost constant. As all experiments were performed using uncoated fused-silica capillaries, an electroosmotic flow directed towards the cathodic end of the capillary was always present and the negatively charged warfarin electrophoretically migrated counter-flow to electroosmosis. However, the net migration of warfarin was always in the same direction as electroosmosis. Table I shows the expected decrease in electroosmosis [17] and the resulting increase in the migration time of warfarin, t_{wf}, as pH is decreased between 9.1 and 6.2. The value of t_{wf} is at a maximum at *ca.* pH 6 and then decreases to become closer to the residence time of the electrolyte in capillary, t_{eo}, as the negative charge of warfarin decreases down to pH 6. In this whole range of pH, 5-chlorowarfarin migrates faster than warfarin because of its presumed slower absolute electrophoretic mobility. Nevertheless, at pH 4.7, chlorowarfarin and warfarin comigrate, which suggests that the unknown acidic p*K* of chlorowarfarin is lower than that of warfarin. As shown in Table I, the migration times of warfarin

TABLE I

EFFECT OF pH VARIATION

Effect of pH on electroosmosis (as evaluated through the migration time of methanol, t_{eo}), migration times of analytes of interest (t_{ClWf} and t_{Wf}) and chiral resolution (as expressed by $(h_m - h_v)/h_v$) of warfarin enantiomers. Migration times separated by the symbol / refer to a pair of enantiomers. All electrolytes contained 8 m*M* Me-β-CD and 2% methanol. Other conditions as in Experimental.

Electrolyte	pH	I (μA)	t_{eo} (min)	t_{ClWf} (min)	t_{Wf} (min)	$(h_m - h_v)/h_v$
20 mM Buffer containing 100 mM NaCl						
Acetate	4.7	68	18.9	21.1	21.1/21.2	0.23
MES[a]	6.2	61	18.4	31.8/32.4	38.2/38.7	0.37
Phosphate	7.0	67	11.1	16.1	18.4/18.8	0.97
TAPS[b]	8.35	59	7.6	9.9	10.6/10.8	0.96
Borate	9.1	61	6.9	8.4	9.0/9.2	0.78
100 mM Phosphate buffer without NaCl						
	7.9	67	8.7	11.5	12.6/12.8	0.98
	8.35	70	7.9	10.2	11.3/11.5	0.99
	8.8	74	7.6	9.7	10.6/10.8	0.98

[a] MES = morpholinoethanesulphonate.
[b] TAPS = N-tris(hydroxymethyl)methyl-3-aminopropanesulphonate.

enantiomers remained very close to each other, and none of these electrolytes gave a baseline chiral resolution. For the sake of convenience and greater significance, it was found better in the present case, as already suggested [18], to express resolution as the ratio $(h_m - h_v)/h_v$, where h_m is the mean peak height and h_v is the height of the valley. This factor gives normalized values between 0 and 1 (baseline resolution). Thus, it is seen that the best resolutions were obtained with-

TABLE II

EFFECT OF ELECTROLYTE IONIC STRENGTH

Effect of electrolyte ionic strength on electroosmosis (as evaluated through the migration time of methanol, t_{eo}), migration times of analytes of interest (t_{ClWf} and t_{Wf}) and chiral resolution (as expressed by $(h_m - h_v)/h_v$) of warfarin enantiomers. Migration times separated by the symbol / refer to a pair of enantiomers. All electrolytes contained 8 m*M* Me-β-CD and 2% methanol. Other conditions as in Experimental.

Electrolyte	I (μA)	t_{eo} (min)	t_{ClWf} (min)	t_{Wf} (min)	$(h_m - h_v)/h_v$
20 mM TAPS buffer (pH 8.35) containing various concentrations of NaCl					
0 m*M*	4	5.05	6.05	6.30/6.35	0.67
30 m*M*	21	6.35	7.85	8.3/8.4	0.90
80 m*M*	47	7.1	9.0	9.65/9.8	0.94
100 m*M*	59	7.65	9.9	10.65/10.8	0.96
125 m*M*	75	7.85	10.3	11.2/11.35	0.985
100 mM TAPS buffer (pH 8.3)					
	26	7.0	8.6	9.0/9.1	0.89
100 mM Phosphate buffer (pH 8.35)					
	70	7.9	10.2	11.3/11.5	0.99

in a 7–8.5 pH range, using phosphate and TAPS buffers.

The effect of pH was further studied in this restricted range with 100 m*M* phosphate buffers without sodium chloride. It appears (Table I) that for similar buffer pH values, concentrations and conductivities, a better resolution was achieved on taking over a phosphate from a TAPS buffer. However, the ionic strength of the phosphate buffer was greater than that of the TAPS buffer, owing to the greater negative charge borne by phosphate ions.

Effect of electrolyte ionic strength

In order to evaluate further the effect of electrolyte ionic strength, another series of experiments was performed at the optimum pH (8.35) with 20 m*M* TAPS buffers containing increasing concentrations of sodium chloride. Table II shows the expected decrease in electroosmotic flow and the concomitant increase in the migration time of warfarin. Indeed, the chiral resolution, expressed as the same ratio, increased drastically as the sodium chloride concentration increased. However, this concentration should not much exceed 125 m*M*, otherwise the electrolyte conductivity would be too high. A still better resolution was produced with a 100 m*M* phosphate buffer of similar conductivity, but about double the ionic strength (*ca.* 275 m*M*). TAPS buffer electrolyte of equal concentration but much lower ionic strength (*ca.* 50 m*M*) gave a much lower resolution (Table II). All these results suggested that ionic strength plays an important role in the differential complexation of warfarin enantiomers, and a 100 m*M* phosphate buffer (pH 8.35) was used subsequently.

Effect of methyl-β-cyclodextrin concentration

The chiral resolutions of warfarin and 5-chlorowarfarin enantiomers were determined with 100 m*M* phosphate buffer electrolytes (pH 8.35) containing Me-β-CD at concentrations varying from 0 to 20 m*M*. Fig. 2 shows some of the resulting electropherograms. Warfarin enantiomers were almost fully separated with Me-β-CD con-

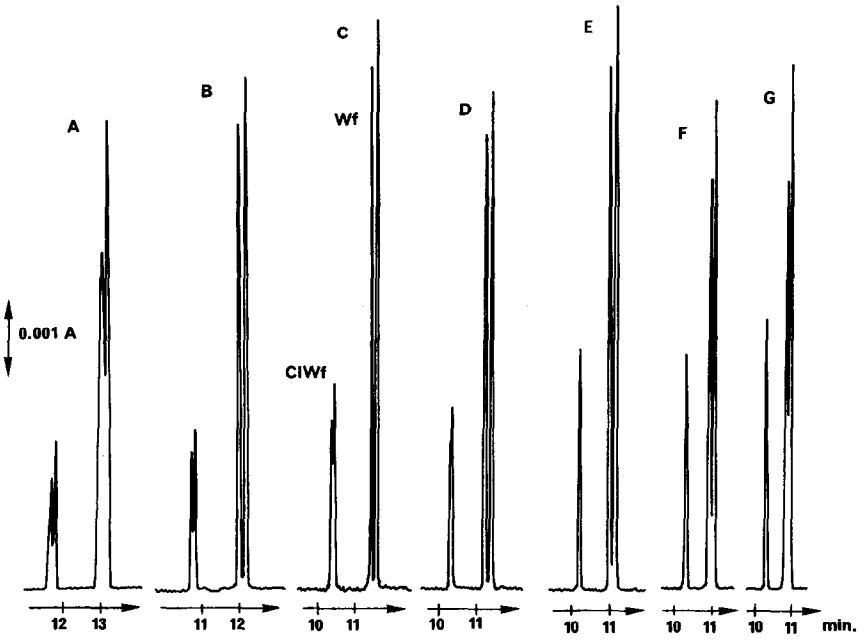

Fig. 2. Effect of Me-β-CD concentration on the chiral resolutions of warfarin (Wf) and 5-chlorowarfarin (ClWf). Electrolytes contained 100 m*M* phosphate buffer (pH 8.35), 2% methanol and the following Me-β-CD concentrations: 2 m*M* (A), 4 m*M* (B), 6 m*M* (C), 8 m*M* (D), 10 m*M* (E), 15 m*M* (F) and 20 m*M* (G). Other conditions as in Experimental. The $(h_m - h_v)/h_v$ chiral resolution ratios obtained were 0.55 (A), 0.985 (B), 0.99 (C), 0.99 (D), 0.95 (E), 0.83 (F) and 0.63 (G).

TABLE III

EFFECT OF METHANOL CONTENT IN ELECTROLYTE

Effect of methanol content on electroosmosis (as evaluated through t_{eo}), migration times (t_{Wf}) and chiral resolution (as expressed by $(h_m - h_v)/h_v$) of warfarin enantiomers for two phosphate buffers (pH 8.35) of different concentrations (20 and 100 m*M*). Migration times separated by the symbol / refer to a pair of enantiomers. All electrolytes contained 8 m*M* Me-β-CD and 2% methanol. Other conditions as in Experimental.

Methanol content (%)	t_{eo} (min)		t_{Wf} (min)		$(h_m - h_v)/h_v$	
	20 m*M*	100 m*M*	20 m*M*	100 m*M*	20 m*M*	100 m*M*
0	6.0	7.3	7.6/7.7	10.1/10.3	0.76	0.98
2		8.0		11.3/11.5		0.99
5	6.7	8.4	8.7/8.8	12.1/12.3	0.81	0.98
10	7.5	9.5	10/10.1	14.2/14.5	0.81	0.985
15	8.1		11.4/11.5		0.77	
20	8.1		11.4/1		0.70	

centrations between 4 and 8 m*M*, the maximum resolution being reached at 6 m*M*. However, almost simultaneously, 5-chlorowarfarin isomers exhibit partial separation with Me-β-CD concentration between 1 and 6 m*M*, the best being at 3 m*M*. As it seemed preferable for a compound intended to act as an internal standard to produce a single peak, it was decided to select a concentration providing a low resolution of 5-chlorowarfarin isomers. Thus, a 8 m*M* concentration of Me-β-CD was considered as optimal.

TABLE IV

EFFECT OF APPLIED VOLTAGE

Effect of applied voltage on current intensity (*I*), electroosmosis (as evaluated through t_{eo}), migration times (t_w) and chiral resolution (as expressed by $(h_m - h_v)/h_v$) of warfarin enantiomers. Migration times separated by the symbol / refer to a pair of enantiomers. Electrolyte composition: 100 m*M* phosphate buffer (pH 8.35)–8 m*M* Me-β-CD, containing 2% methanol. Other conditions as in Experimental.

Voltage (kV)	*I* (mA)	t_{eo} (min)	t_w (min)	$(h_m - h_v)/h_v$
15	45	11.9	16.9/17.1	0.99
20	68	7.9	11.3/11.5	0.99
25	96	5.4	7.9/8.0	0.94
30	142	3.6	5.3/5.4	0.82

Effect of electrolyte methanol content

The electrolyte methanol content was investigated within a 0–20% (v/v) range for two phosphate buffers (pH 8.35), 20 and 100 m*M*, respectively. All the electrolytes contained 8 m*M* Me-β-CD, according to the preceding study. The presence of methanol in the electrolyte was of paramount importance in the separation of propranolol enantiomers by FSCE with β-CD [11]. For both buffer concentrations, a slight improvement in resolution was noticed with 2–5% (v/v) methanol added to the aqueous electrolyte (Table III). It is thought that a small amount of methanol improves the analyte solubility in the electrolyte during the migration. Nevertheless, the addition of methanol should remain very limited to avoid both an increased separation time owing to slower electroosmosis [17] and a loss of resolution due to weaker inclusion complexation.

In addition, Table III confirms the prominent role played by the electrolyte ionic strength insofar as it highlights that, for about equal electroosmotic flows, the chiral resolution of warfarin isomers was far better with a 100 m*M* phosphate buffer containing 2% (v/v) methanol than with a 20 m*M* phosphate buffer containing 15–20% methanol.

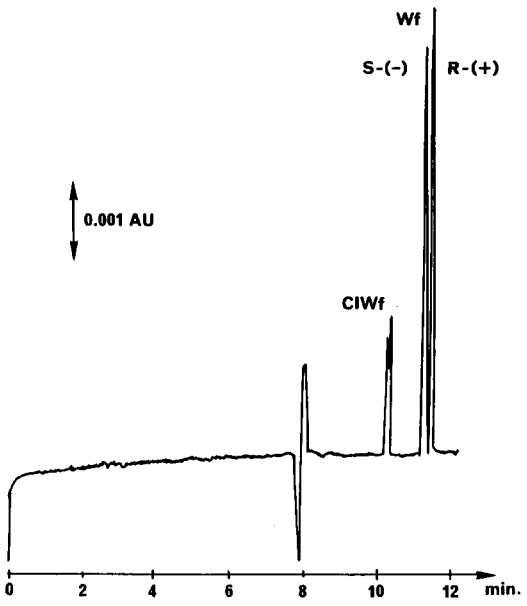

Fig. 3. Separation of warfarin enantiomers under optimized operating conditions. Capillary, fused silica, 72 cm × 50 μm I.D. (50 cm to detection window); electrolyte, 100 m*M* sodium phosphate buffer (pH 8.35); 8 m*M* Me-β-CD–methanol (98 : 2, v/v); applied voltage, 20 kV (*I* = 70 μA); temperature, 25°C; UV detection, 310 nm; hydrodynamic injection time, 1 s. Peaks: ClWf = 5-chlorowarfarin; Wf = warfarin.

Effect of applied voltage

Finally, the effect of voltage on the chiral resolution was studied between 15 and 30 kV, keeping the previously selected optimal conditions for the other operating parameters. It was found (Table IV) that an increase in applied voltage from 15 to 30 kV causes more than a three-fold increase in current intensity and electroosmosis. This behaviour clearly indicates a heating of the core of the electrolyte in the capillary over this range of voltages, which can be mainly understood as a result of the high electrolyte ionic strength required for proper chiral discrimination. Simultaneously, the chiral resolution is drastically reduced. So, the best compromise between resolution and analysis time seemed to use a 20-kV voltage.

The full optimized operating conditions are given in Fig. 3. The two enantiomer peaks were identified by successively injecting two racemic mixtures enriched in each enantiomer, which were obtained by fractional crystallization [19]. The order of migration observed suggests that the *S*-(−)-isomer is more strongly complexed by the cyclodextrin than the *R*-(+)-isomer, which is in agreement with the retention order previously observed in liquid chromatography [7]. The separation efficiency is of the order of $2.5 \cdot 10^5$ theoretical plates. The repeatabilities were determined to be 0.2% R.S.D. (n = 5) for migration times, 9 and 8.5% R.S.D. for absolute peak-heights and corrected peak areas (peak area divided by migration time), respectively, and 2.0 and 2.1% R.S.D. for the relative peak heights (solute to internal standard peak-height ratios) and relative corrected peak areas (solute to internal standard corrected peak-area ratios), respectively.

Determination of warfarin enantiomers in plasma samples

The determination of warfarin enantiomers in human plasma samples was performed using the optimized conditions given in Fig. 3. It was first ascertained that a blank plasma gave no interfering peak within the range of migration times of warfarin enantiomers. A study of repeatability (n = 6) with a veal plasma sample spiked with 2 mg/l warfarin and subjected to the extraction protocol described was conducted and yielded the following results: 0.3% R.S.D. for migration times, 1.1 and 1.4% R.S.D. for absolute peak heights and corrected peak areas, respectively, and 3.8 and 5.2% R.S.D. for relative peak heights and peak areas, respectively. Four patients under warfarin therapy (A, B, F and L) were then tested. A typical electropherogram is shown in Fig. 4. The migration order of *S*-(−)- and *R*-(+)-warfarin was as previously stated.

The ratios of peak heights of each warfarin enantiomer to 5-chlorowarfarin racemate were measured. The results, given in Table V, show that the total racemic warfarin concentration in plasma differed only slightly from patient to patient, with a mean value being close to 2.0 mg/l. It must be mentioned that the dose of warfarin administered to the patients was 10 mg per day, and that these patients were under treatment for sev-

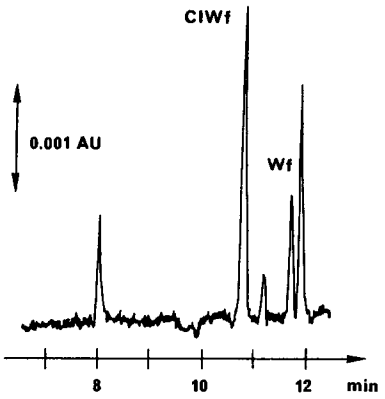

Fig. 4. Electropherogram of a plasma sample of a patient (F) under warfarin (Wf) therapy. Sample preparation as in Experimental. Operating conditions and identification as in Fig. 3. The additional unlabeled peaks were not identified. The total warfarin concentration determined was 2.0 mg/l with an *S*/*R* enantiomeric ratio of 34:66. ClWf = 5-chlorowarfarin.

eral months and can be considered to be at a "steady state". The enantiomeric compositions obtained for different patients are a little more scattered but indicate that the *S*-(−)-isomer is metabolized faster. All these results agree with previous ones [8].

Finally, under these conditions, the minimum detectable concentration (MDC) in plasma, corresponding to a signal three times greater than the background noise, was evaluated as 0.2 mg/l ($6.5 \cdot 10^{-7}$ *M*) for each warfarin enantiomer. The linearity range was estimated to two orders of magnitude, from this limit up. It can be noted that, without the preconcentration afforded by the extraction step, the MDC would be only 4 mg/l ($1.3 \cdot 10^{-5}$ *M*). Under the present conditions, increasing the injection time above 1 s resulted in enlarged peaks and an unacceptable loss of resolution. However, no attempt to further decrease MDC by dissolving the serum extracts in a low conductivity medium and by using the electrokinetic mode of sample introduction was investigated.

CONCLUSION

Capillary electrophoresis has proven to be a valuable alternative to liquid chromatography for the determination of warfarin enantiomers in plasma samples. A baseline resolution is obtained using a methylated β-cyclodextrin-containing electrolyte in under 15 min with a remarkable efficiency. However, it would be interesting to improve the limit of detection by a preconcentration electrophoretic step, because in the present

TABLE V

DETERMINATION OF WARFARIN ENANTIOMERS IN HUMAN PLASMA

Standards were veal plasmas spiked with the warfarin (Wf) concentrations mentioned and a fixed amount of 5-chlorowarfarin (ClWf). Human plasmas containing unknown amounts of warfarin were spiked with the same amount of 5-chlorowarfarin (see Experimental).

	Total Wf enantiomer/ClWf peak-height ratio	Toral racemic Wf concentration (mg/l)	Enantiomer/ClWf peak-height ratio		Enantiomeric composition (%)	
			S-(−)-Wf	*R*-(+)-Wf	*S*-(−)-Wf	*R*-(+)-Wf
Standards						
0	0	0				
1	0.55	1.0			50	50
2	1.15	2.0			50	50
3	1.65	3.0			50	50
Plasmas						
A	1.20	2.1	0.48	0.72	40	60
B	1.15	2.0	0.46	0.69	40	60
F	1.13	2.0	0.38	0.75	34	66
L	1.02	1.8	0.34	0.68	33	67

state this limit is *ca.* ten to twenty times higher than that recently reported, using fluorescence detection coupled to a chromatographic column of conventional size [8]. We will report soon on the determination of inclusion complex formation constants of warfarin and 5-chlorowarfarin with Me-β-CD by various methods, including capillary electrophoresis. It is thought that the availability of these data will serve to better elucidate the chiral recognition mechanisms.

ACKNOWLEDGEMENTS

The authors thank Dr. P. Fabiani, Honorary Professor of Toxicology at the University René Descartes, for the preparative separation of *S*-(−)- and *R*-(+)-warfarin, enabling the identification of both enantiomers in the electropherograms. Dr. E. Kolossa from Waker-Chemie GmbH is also gratefully acknowledged for the gift of the modified β-cyclodextrin.

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