Simultaneous Chiral Separation of Leucovorin and its Major Metabolite 5-Methyl-tetrahydrofolate by Capillary Electrophoresis Using Cyclodextrins as Chiral Selectors: Estimation of the Formation Constant and Mobility of the Solute-Cyclodextrin Complexes

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Key Words

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Summary

Capillary electrophoresis with an electrolyte containing cyclodextrin was investigated for the simultaneous separation of the diastereoisomers of 6R,S-leucovorin and its active metabolite 6R,S-5-methyl-tetrahydrofolate. α, β and γ-cyclodextrin separated the diastereoisomers of 5-methyl-tetrahydrofolate, while only γ-cyclodextrin was found to be effective for the chiral separation of leucovorin. The effect of γ-cyclodextrin concentration was investigated, and subsequently a curve-fitting analysis for the quantitative estimation of the binding constants was attempted. The binding constants were found to be very small, in the range 2–4 M^{-1}. Although the interaction between γ-cyclodextrin and the tetrahydrofolates is weak, the high efficiency of capillary electrophoresis and the use of high concentrations of γ-cyclodextrin allow baseline chiral separation of the diastereoisomers of leucovorin and 5-methyl-tetrahydrofolate. Changes in temperature exert differing effects on the separations of leucovorin and 5-methyl-tetrahydrofolate; higher temperatures improved the separation of leucovorin diastereoisomers but reduced the resolution of 5-methyl-tetrahydrofolate diastereoisomers. The effects of urea and buffer salt concentrations and of buffer pH were also investigated. Capillary electrophoresis with γ-cyclodextrin was used to analyse plasma samples spiked with clinically-relevant levels of leucovorin and 5-methyl-tetrahydrofolate. Resolution of these compounds in ultrafiltered plasma was demonstrated, but detection sensitivity was not adequate for the routine use of this method for the determination of leucovorin and 5-methyl-tetrahydrofolate in plasma. In addition, a simple technique to reverse the elution order of ionic stereoisomers was demonstrated. By adding a cationic surfactant into the buffer and reversing the separation potential, the elution order of the diastereoisomers of leucovorin and 5-methyl-tetrahydrofolate was reversed.

Introduction

Capillary electrophoresis (CE) has become a popular analytical technique, with numerous fundamental studies and applications in a wide range of research fields being reported [1, 2]. One interesting area of application is in chiral separations. The high efficiency of CE is expected to allow the resolution of optical isomers based on a slight stereoselectivity which may not result in a separation using conventional high performance liquid chromatography (HPLC) methods. So far several methods have been reported for direct chiral resolution by CE [3], such as the use of chiral ternary complexes [4], chiral surfactants and mixed micelles [3], and cyclodextrins [3]. Cyclodextrins (CDs) are cyclic oligosaccharides containing rings of α-(1,4)-linked D-glucose moieties. CDs can include compounds into their central cavity, with the stability of this inclusion complex depending on the fit between the compound and the cavity. The inclusion phenomenon may be stereoselective because of the chirality of the constituent glucose molecules. CDs are widely used for chiral HPLC separations via immobilisation onto a stationary phase [5] as well as by addition into the mobile phase [6]. By selecting the size of CD used, a wide range of the optical isomers can be separated.

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Guttman and co-workers reported the application of immobilised CDs to chiral separations in CE where the CD was incorporated within a polyacrylamide gel matrix, allowing the resolution of enantiomers of dansylated amino acids [7]. Chiral separation of ionic isomers can also be achieved by simply adding CD into the running buffer solution without immobilization. Chiral separations of ephedrine and related compounds [8], tryptophan [9] and β-blockers [10] have been shown using coated capillaries and an acidic buffer containing CD, while Snopek et al. [11] reported chiral separations with CDs using cellulosic additives and acidic electrolytes to minimise electroosmosis. The stereoselectivity of CDs has also been used in conjunction with ionic micelles to achieve resolution of ionic and also neutral stereoisomers [12, 13]. Since in this mode CD affects not only the electrophoretic migration of a solute but also its distribution into the micelles, the chiral separation mechanism is more complex than with CD additives alone. A number of publications have reported attempts to quantitate the binding of stereoisomers to chiral selectors in CE [7, 14, 15]. Wren and Rowe [14] showed that the optimum concentration of chiral selector for maximising the mobility difference between two stereoisomers may be related in a simple fashion to the strength of the binding interactions.

Leucovorin (LV) is a reduced foliate which is used in cancer chemotherapy to treat and prevent host toxicity due to the administration of supralethal doses of methotrexate [16]. LV is administered as a 1 : 1 mixture of (6R)- and (6S)-iastereoisomers. (6S)-LV is the pharmaceutically active form, and is rapidly converted *in vivo* to an active metabolite, 5-methyltetrahydrofolate (ME). The pharmacokinetic behaviour also shows stereoselectivity; the half-lives of (6S)-LV and ME are significantly shorter than that of (6R)-LV. Recently, an achiral/chiral coupled column HPLC system was developed for the direct resolution of LV and ME [17]. The achiral (phenyl silica) column was incorporated to compensate for the poor separation between (6R)-LV and (6R)-ME on the chiral (bovine serum albumin) column. This system was further modified by including a column-switching concentration step to allow the determination of low levels of the tetrahydrofolates [18]. However, these systems are not easy to use and a simpler method is desirable.

In this paper, the use of CE with CD additives for the simultaneous chiral separation of LV and ME is described. The effect on the separation of the size and concentration of CD, temperature, buffer pH and ionic strength, and concentration of urea were systematically investigated. The formation constants and electrophoretic velocities of the LV- and ME-CD complexes were estimated, and some of the difficulties of measuring binding when using high concentrations of chiral selector are discussed. The analysis of LV and ME in plasma samples is demonstrated. In addition, a simple method to reverse the elution order of ionic stereoisomers is described.

Experimental

CE separations were carried out using an Applied Biosystems 270A-HT (Foster City, CA, USA) integrated CE system. Fused silica capillaries of 370 μm external diameter, 51 μm internal diameter were obtained from Polymicro Technologies (Phoenix, AZ, USA) and cut into 72 cm lengths. The polyamide coating on the capillary was removed 22 cm from one end to provide a detection window, giving an effective separation length of 50 cm. Injections were made by application of a vacuum at the capillary outlet. Detection was by on-capillary UV absorbance measurements at a wavelength of 289 nm, with a rise time of 0.5 s or 2.0 s. Data was analysed using a Spectra-Physics (San Jose, CA, USA) Datajet integrator. Methanol or acetone was used as a tracer of electroosmotic flow.

Racemic LV, (6R)-LV and (6R)-ME as calcium salts, and (6S)-LV and (6S)-ME were provided by Eprova AG (Schaffhausen, Switzerland). Ascorbic acid (AsA) was purchased from BDH Chemicals (Toronto, Canada). Sodium dodecyl sulfate (SDS), urea and α-CD were purchased from Aldrich Chemical Company (Milwaukee, WI, USA). β-CD was purchased from ICN (Cleveland, OH, USA). γ-CD was purchased from Advanced Separation Technologies Inc. (Whippany, NJ, USA). Sodium (-)-6-methoxy-α-methyl-2-naphthaleneacetate, used as an internal standard, and cetyltrimethylammonium bromide (CTAB) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Racemic LV, (6R)-LV, (6S)-LV and (6R)-ME were dissolved in a 1 mg mL^{-1} aqueous solution of AsA, to prepare stock solutions. (6S)-ME was dissolved in 1 mg mL^{-1} AsA aqueous solution, the pH of which was adjusted to 7 by adding 1 M NaOH. AsA was added to the sample solutions to prevent oxidation of the tetrahydrofolates. All stock solutions were stored at -20 °C.

Plasma samples were prepared for analysis by the following procedure: 900 mg of urea was added to 1.5 mL of plasma spiked with LV and ME (to which 5 mg mL^{-1} of AsA had previously been added) and the sample was vortexed for 1 min. 1 mL of the sample solution was transferred to a Centrifree ultrafilter (Amicon, Danvers, MA, USA). This was centrifuged for 40 min at 2000 g. 10 μL of 40 mM (-)-6-methoxy-α-methyl-2-naphthaleneacetate was then added to 200 μL of the filtrate. The urea denaturation leads to complete recovery for each diastereoisomer [19, 20], although addition of less urea lead to an incomplete, stereoselective release of LV and ME.

Results and Discussion

Migration Behaviour Using Achiral CE

It is well known that, unlike enantiomers, diastereoisomers are sometimes separable under achiral chroma-

tographic conditions. The same is true in CE; for example, the diastereoisomers of L-buthionine-(R,S)-sulfoximine were resolved under achiral CE conditions [21]. Therefore, at first, the diastereomeric separations of LV and ME were attempted without the use of chiral selectors.

Table I shows the effect of addition of the anionic surfactant SDS and the cationic surfactant CTAB upon the migration time and the capacity factor of LV, ME and AsA. Sudan III was used as a tracer of the micellar migration. The pH of the buffer was kept at 7. At this pH, LV and ME both carry two negative charges. Using SDS with a positive voltage applied at the injection end of the capillary, the overall migration of the tetrahydrofolates is towards the cathode. The migration times increased by increasing the SDS concentration because of greater association of the solutes with the micelles, and because of a slight decrease in electroosmotic flow. The elution order of LV and ME reversed by adding SDS into the buffer compared to the separation with no added surfactant, indicating that ME is incorporated by the micelle more than LV.

Using CTAB, the electroosmotic flow is toward the anode because the cationic surfactant adsorbs onto the inner capillary wall and reverses the surface charge [22]. In this mode, negative voltage was applied at the injection end. By increasing the CTAB concentration, both the electroosmotic flow, and the solute mobility towards the cathode increased. As these two opposing motions balance each other, the migration times decreased with increasing CTAB concentration from 5 mM to 20 mM, followed by a slight increase in migration time with the further increase in surfactant concentration up to 50 mM. The capacity factors calculated by Eq. (7) in Ref. [23] were much larger with CTAB than with SDS because the interaction of these anionic solutes is preferred with the cationic micelle rather than with the anionic micelle [24, 25].

Although LV, ME and AsA were easily separated under achiral conditions, resolution of the diastereoisomers was not achieved. Further modifications of the separation electrolyte such as changing pH between 5 and 9 and adding 10 % methanol or 10 and 15 % isopropanol did not give diastereomeric separations. This is perhaps not too surprising, since the chiral centres of LV and ME are located far from each other in the molecule, and thus their mutual interactions may be very weak.

Chiral Separations Using CD Additives

First the effect of the type of cyclodextrin was investigated, since selection of the size of CD is usually an important point for chiral separations [5]. Figure 1 shows separations using α-, β- and γ-CD. Urea was added together with β- and γ-CDs to increase their solubilities, but was not added with α-CD because, on the contrary, urea decreases its solubility [26]. CD was added to the electrolyte in the inlet reservoir only, thus the volume used which contained CD was as small as 4

Table I Migration times and capacity factors (k') using SDS and CTAB surfactant additives. EOF represents electroosmotic flow. CE conditions: capillary, 72 cm × 51 μm i.d. (50 cm to the detector); buffer, sodium phosphate (pH 7, I = 0.04); voltage, + 20 kV with SDS and – 20 kV with CTAB; temperature, 25 °C.

Migration time/min of	SDS concentration/mM			CTAB concentration/mM		
	0	80	200	5	20	50
LV	7.74	9.76	11.21	14.79	8.74	9.36
ME	7.54	10.00	11.39	21.71	10.10	10.61
AsA	7.49	9.69	10.81	5.54	4.27	4.44
EOF	4.61	4.88	5.00	9.66	5.98	5.84
Sudan III		19.30	32.00	26.11	10.80	11.39
k' of LV	0	0.29	0.40	4.23	6.44	7.96
k' of ME	0	0.43	0.50	18.23	23.75	24.33

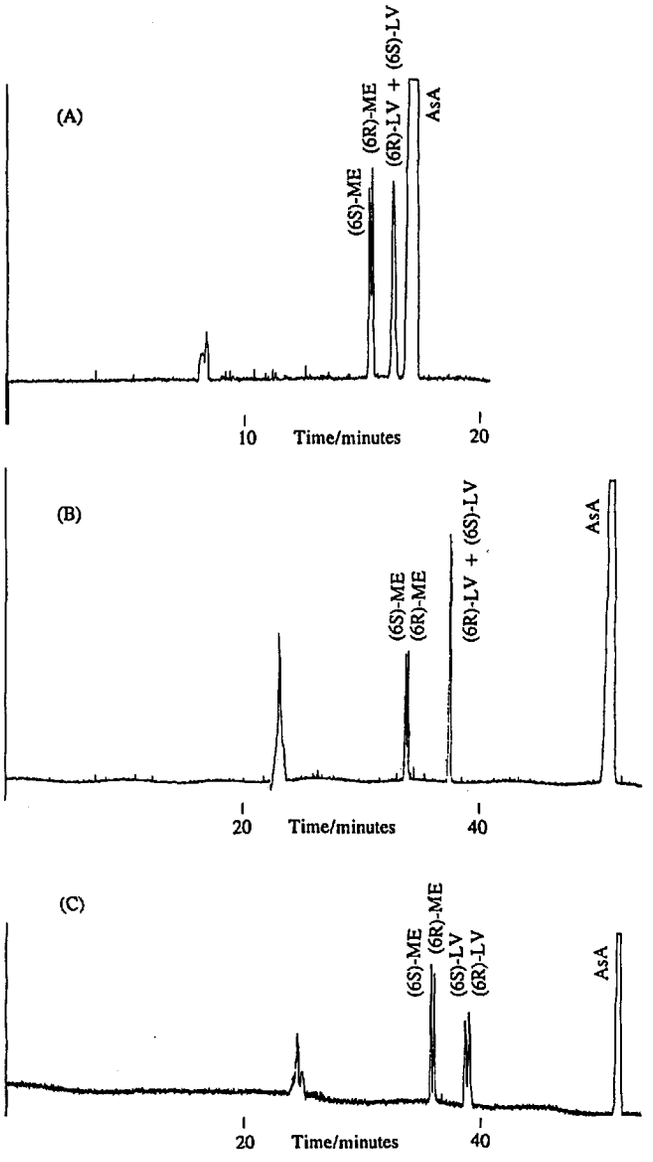

Figure 1

Separation of LV and ME using α-, β- and γ-cyclodextrin. CE conditions: capillary, 72 cm × 51 μm i.d. (50 cm to detector); buffer, sodium phosphate (pH 7, I = 0.1) containing (A) 0.1 M α-CD, (B) 0.2 M β-CD and 8 M urea, (C) 0.2 M γ-CD and 6 M urea; voltage, 20 kV; temperature, 25 °C.

mL. From an economic viewpoint, this is particularly advantageous when using an expensive chiral additive such as γ-CD. When α-CD (Figure 1A) and β-CD (Figure 1B) were added into the buffer the ME diastereoisomers were separated, but not the LV diastereoisomers. When γ-CD was added (Figure 1C), the diastereoisomers of both LV and ME were separated. Unlike bovine serum albumin-based chiral HPLC [17, 18], there was no overlap between LV and ME. Separations of LV and ME are achieved with 0.2 M γ-CD over a urea concentration range of 2 to 8 M (data shown in Figure 9 later), thus the difference between the separations with β- and γ-CD seen in Figure 1 are certainly due to the type of CD used, and are not related to differences in the urea concentration.

The fact that ME is separated with each of the cyclodextrins while chiral resolution of the LV diastereoisomers is only achieved using γ-CD is rather surprising, given the rather small structural difference between the two compounds. It suggests that the nature of the complex formed between ME and γ-CD is different from that formed between LV and γ-CD. The separation of ME by all three CDs suggests that the complex is not due to an inclusion of ME into the cyclodextrin cavity, but to an interaction on the surface of the CD. We speculated that if this were the case, then glucose added to separation buffer instead of CD might also interact with ME and LV. LV and ME were analysed with 6 M urea and 1 M D-glucose as buffer additives, and the results compared with a separation with just 6 M urea added. Without glucose, LV had a longer migration time than the AsA marker, and ME eluted at the same time as AsA. The separation with glucose gave the order of migration times ME < LV < AsA, however, there was no trace of chiral resolution for ME or LV, and the reduction in velocity for both compounds was proportionally similar when compared to their migration velocities without glucose. Thus, although the relative reduction in the velocities of the tetrahydrofolates was greater than that of AsA, there is no indication of differences between the interactions of LV and ME with glucose, and no measurable stereoselectivity.

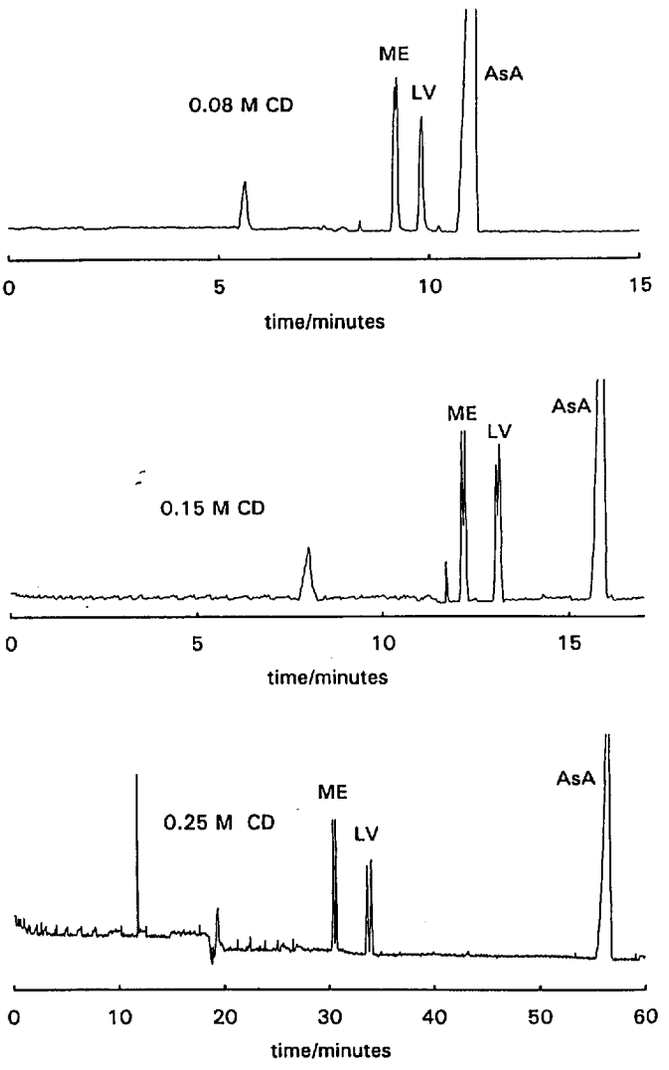

Figure 2

Separation of LV and ME using differing concentrations of γ-CD. CE conditions: capillary, 72 cm × 51 μm i.d. (50 cm to detector); buffer, sodium phosphate (pH 7, I = 0.1) containing 0.08 M γ-CD (upper trace), 0.15 M γ-CD (middle trace) and 0.25 M γ-CD (lower trace), and 6 M urea; voltage, 30 kV; temperature, 40 °C.

Effect of γ-CD Concentration

Table II lists the effect of γ-CD concentration upon the electrophoretic velocities of LV, ME, AsA and the electroosmotic flow, and the diastereometric resolution. The figures are aveages of between two and four determinations at each concentration. A negative sign for the electrophoretic velocity indicates movement in a direction opposite to that of the electroosmotic flow. Figure 2 shows typical electropherograms with 0.08, 0.15 and 0.25 M added γ-CD. It can be seen that the diastereomeric resolution of LV and ME increased with increasing γ-CD concentration. For both LV and ME, the S-isomer is eluted before the R-isomer.

By adding γ-CD into the buffer, the order of migration times is changed from AsA = Me < LV to Me < LV < AsA. With increasing γ-CD concentration, both the velocity of electroosmotic flow, v_{eo}, and the electrophoretic velocity of the solutes, v_{ep}, towards the anodic end are decreased. The observed decrease in v_{eo} and v_{ep} can be ascribed in part to the increase in the solution viscosity caused by addition of γ-CD, and if the solute forms a complex with γ-CD there is a corresponding increase in size, which will contribute to a decrease in v_{ep} [14]. v_{ep} can be described in terms of the velocity of the free solute, v_{epS}, and that of the inclusion complex, v_{epCS} by

$$v_{ep} = ([S]/([S] + [CS]))\, v_{epS} + ([CS]/([S] + [CS]))\, v_{epCS} \quad (1)$$

where [S] and [CS] represent the concentrations in the buffer of the free solute and the inclusion complex respectively. Assuming that γ-CD makes a 1 : 1

Table II Effect of γ-CD concentration on the effective velocity and resolution of the tetrahydrofolates. * indicates resolution was too small to be measured. CE conditions: capillary, 72 cm × 51 μm i.d. (50 cm to the detector); buffer, sodium phosphate, pH 7, I = 0.1, containing γ-CD and 6 M urea; voltage, 30 kV; temperature, 40 °C.

[CD]/ M	v_{effRLV}/ cm/min	v_{effSLV}/ cm/min	R_{LV}	v_{effRME}/ cm/min	v_{effSME}/ cm/min	R_{ME}	v_{effAsA}/ cm/min
0.0	- 5.981	- 5.981	*	- 5.842	- 5.842	*	- 5.842
0.02	- 5.260	- 5.260	*	- 5.034	- 5.034	*	- 5.407
0.05	- 4.464	- 4.540	*	- 4.129	- 4.114	0.41	- 4.831
0.08	- 3.837	- 3.828	*	- 3.508	- 3.476	0.42	- 4.413
0.10	- 3.163	- 3.142	0.44	- 2.844	- 2.814	0.89	- 3.760
0.15	- 2.417	- 2.393	0.76	- 2.139	- 2.114	0.89	- 3.080
0.20	- 1.632	- 1.611	1.07	- 1.418	- 1.398	1.08	- 2.255
0.25	- 1.180	- 1.163	1.27	- 1.012	- 0.994	1.12	- 1.768

inclusion complex with the tetrahydrofolates, the formation constant, K, is defined as

$$K = [CS]/[C]\,[S] \quad (2)$$

where [C] is the free CD concentration. Putting Eq. (2) into (1), the following expression is obtained,

$$v_{ep} = (v_{epS} + v_{epCS}\,K[C])/(1 + K[C]) \quad (3)$$

v_{epS} is the solute velocity with no added CD, and so is easy to measure. v_{epCS} is a function of the limiting solute velocity at a sufficiently high CD concentration for the analyte to be (almost) completely complexed. However, to relate the values of v_{epS} and v_{epCS} thus measured, a correction must usually be made for the effects other than binding on the electrophoretic velocity (e.g. increase in solution viscosity) which occur upon addition of the chiral selector. The addition of a non-ionic substance to an electrolyte solution causes a relative fall in conductivity which is smaller than the relative increase in solution viscosity, and this relative difference increases as a function of the volume-fraction of the non-conducting additive [27]. We investigated what would be the most suitable correction factor to use in this separation, considering the viscosity, the electroosmotic velocity, the current and the velocity of the AsA as a marker compound, all measured relative to their values with no added CD. The relative viscosity as a function of CD concentration was determined by measuring the migration time for a marker compound injected into the CE system and sucked through the capillary by a controlled pressure of 20″ Hg. The accuracy of this measurement was confirmed by measuring the relative viscosity of aqueous sucrose solutions, for which accurate tabulated data is available [28]. It was found that there was a linear relation between the measured relative viscosity for the sucrose solutions (y) and literature values (x) for solutions varying from 0 to 35 % sucrose ($y = 1.008x - 0.007$, $r^2 = 0.9995$; 10 data points). Figure 3 shows the viscosity ratio (determined as described above), the electroosmotic velocity ratio and the AsA velocity ratio, plotted as a function of the current ratio over a range of γ-CD concentration from 0 to 0.25 M. It can be seen that there is a good linear relationship between the current and AsA velocity data ($y = 0.93x + 0.07$, $r^2 = 0.998$). However, both the pressure-driven velocity and electroosmotic velocity measurements, whilst in good agreement with each other, show (as expected [27]) a considerable positive deviation which is most pronounced at higher CD concentrations. Thus in this case relative viscosity measurements are not a suitable basis for calculating corrected velocities, and a direct measure of the reduction in ionic mobilities such as current or the velocity of a marker compound are to the preferred. On the basis of this data, the AsA velocity was used to compensate for the viscosity increase on addition of CD, since AsA was present in each analysis as a preservative. Thus the electrophoretic velocities, v′, normalised for viscosity effects, are given by

$$v'_{ep} = (v_{epAsA\,(0)}/v_{epAsA})\,v_{ep} \quad (4)$$

$$v'_{epCS} = (v_{epAsA\,(0)}/v_{epAsA})\,v_{epCS} \quad (5)$$

where v_{epAsA} represents the electrophoretic velocity of AsA at a given γ-CD concentration, and $v_{epAsA\,(0)}$

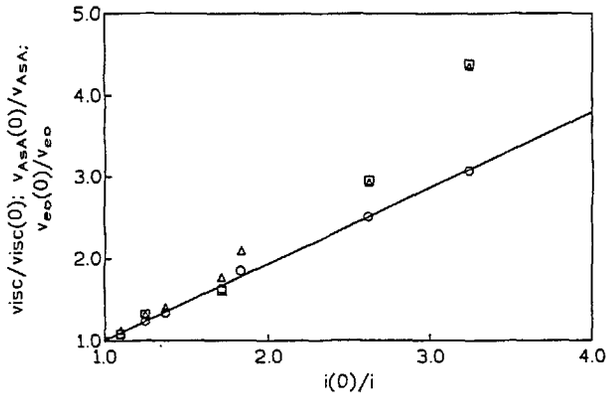

Figure 3

Relative increase in viscosity (□), relative decrease in electroosmotic flow velocity (Δ) and relative decrease in AsA velocity (○) plotted against relative decrease in current, measured over a range of [CD] from 0 to 0.25 M, all values relative to those measured with no added CD in the solution (buffer, sodium phosphate, pH 7, I = 0.1, with 6 M urea; temperature, 40 °C). Electrophoretic velocity measurements made at 30 kV; pressure-driven measurements at a vacuum of 20″ Hg.

represents the velocity observed without γ-CD, v values are those measured, and the subscripts epS and epCS refer to the free and complexed solute respectively. Consequently, the association constant and the corrected electrophoretic velocity of the inclusion complex can be found using the following equation

$$v'_{ep} = (v_{epS} + v_{epCS} K [C])/(1 + K [C]) \quad (6)$$

At a suitably high CD concentration, further addition of CD gives no change in v'_{ep} and this limiting value can be taken as the velocity of the solute-CD complex, thus K may be calculated directly [14]. However, for LV and ME, the limiting value of v'_{ep} had still not been reached at [γ-CD] = 0.25 M. Thus Eq. (6) could not be solved directly for K, and instead we used a non-linear least-squares curve-fitting procedure to obtain estimates for both K and v'_{epCS} Equation (6) was fitted to data over a range of [γ-CD] from 0 to 0.25 M (26 data points for LV, 22 data points for ME) by use of the program MINSQ (MicroMath Scientific Software, Salt Lake City, UT, USA). The concentration of each diastereoisomer in the sample solution was about 45 μM, which is much smaller than the CD concentration in the buffer, so the total CD concentration can be taken as the free CD concentration.

Optimised fittings of Eq. (6) for LV and ME are shown in Figure 4. Table III lists the estimated K and v'_{epCS} values. It should be noted that in spite of the apparently excellent fits of the curves to the experimental data as judged by eye, the standard deviations for K and v'_{epCS} values are quite high. This comes about because of the ability of a change in K to be compensated for in large degree by a change in v'_{epCS}, resulting in a minimal change in the overall sum of squares. In addition, small errors in the determination of v_{epS} lead to relatively large changes in the parameter estimates, and any error in the velocity correction using v_{epAsA} will also lead to erroneous estimates of the parameters. We attempted to perform spectrophotometric measurements of the formation constants of the complexes to obtain in independent estimate of their values, but unfortunately reliable measurements could not be obtained, perhaps because of the weakness of the interactions. Although for the above reasons the estimates for K and v'_{epCS} given here must be considered as approximate values, we consider that the nonlinear curve-fitting procedure has its merits in that even very small values of K can be estimated, and knowledge of v'_{epCS} is not required. The values of K and v'_{epCS} for LV and ME are significantly different. However, a difference greater than the standard deviation is not found between the values of K or v'_{epCS} for the pairs of stereoisomers of LV or ME. Nevertheless, it is interesting to note that for LV there is a relatively large difference between the v'_{epCS} values. One can speculate that in this case chiral separation occurs at least in part due to a difference in the electrophoretic velocities of the complexed species.

Assuming equal values of v'_{epCS}, the CD concentration needed to achieve the maximum velocity difference between two enantiomers is given by [14]

$$[CD] = (K_1 K_2)^{-1/2} \quad (7)$$

A plot of the velocity difference (corrected using v_{epAsA}) between the stereoisomers of ME and of LV as a function of [g-CD] is shown in Figure 5. It can be seen that for LV a maximum has not been reached at g-CD concentration of 0.25 M, while for ME a maximum may occur around [g-CD] = 0.2 M, although the

Table III Values of K and v'_{epCS} estimated using least-squares curve-fitting to Eq. (6).

Sample	K/M^{-1}	$v'_{epCS}/cm\ min^{-1}$
S-LV	2.21 ± 0.18	- 0.03 ± 0.35
R-LV	2.26 ± 0.19	- 0.29 ± 0.33
S-ME	3.85 ± 0.22	- 0.69 ± 0.18
R-ME	3.71 ± 0.20	- 0.71 ± 0.17

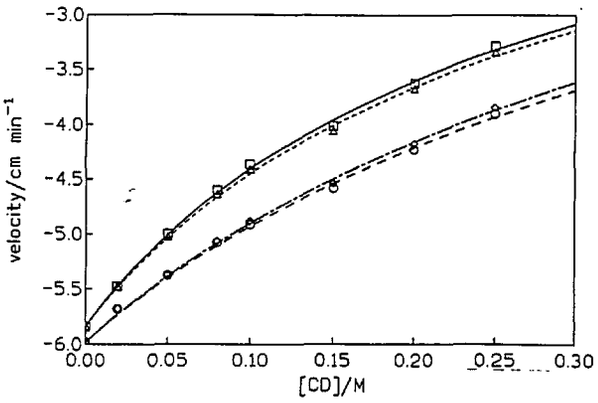

Figure 4

Best-fit lines for modelling of Eq. (6), with data points marked for average values of v'_{ep} for □ = R-ME, Δ = S-ME, ◊ = R-LV, o = S-LV. CE conditions: capillary, 72 cm × 51 μm i.d. (50 cm to detector); buffer, sodium phosphate (pH = 7, I = 0.1) containing γ-CD and 6 M urea; voltage, 30 kV; temperature, 40 °C.

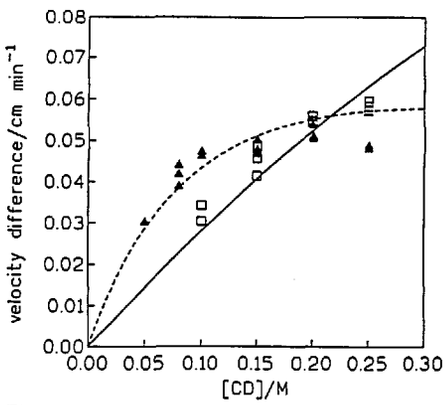

Figure 5

Velocity difference (corrected for viscosity effects) between the diastereoisomers of LV (□) and of ME (▲) as a function of [CD]. Lines represent velocity differences calculated using the estimated formation constants and complex velocities shown in Table III.CE conditions: capillary, 72 cm × 51 μm i.d. (50 cm to detector); buffer, sodium phosphate (pH = 7, I = 0,1) containing γ-CD and 6 M urea; voltage, 30 kV; temperature, 40 °C.

accuracy of such an estimate is poor. From Eq. (7), this would indicate an average value for the formation constant for the ME complexes of around 5 M^{-1}, and for LV of < 4 M^{-1}. This lends some degree of support for the estimates for K shown in Table III. However, the estimates for v'_{epCS} suggest that for LV, the velocities of the diastereomeric complexes may be different, and so for LV the use of Eq. (7) may not be valid. The lines in Figure 5 represent the calculated velocity difference, the velocities being determined using Eq. (6), taking the estimates for K and v'_{epCS} shown in Table III. For ME, with similar values for v'_{epCS} for each stereoisomer, the calculated difference exhibits a maximum around [CD] = 0.25 M, as would be expected from Eq. (7). The behaviour is quite different when there is a significant velocity difference between the complexes, and at high CD concentrations, rather than tending to zero, the velocity difference between the solute enantiomers simply becomes equal to the difference in the velocity of the complexes. Here, the parameter values calculated for LV suggest that a velocity difference between the complexed species is the main driving force for the separation. However, for LV, the velocity difference data obtained up to a maximum g-CD concentration of 0.25 M is not adequate to confirm or contradict this possibility. Measurements were attempted with 0.32 M g-CD, but at this concentration precipitation occurred in the buffer reservoirs and so measurements at such high CD concentrations were abandoned.

The calculated values for K listed in Table III are extremely small. In HPLC where β-CD has been used as a chiral additive in the mobile phase, the formation constants of CD-analyte complexes of several chiral solutes were found to range between 43 and 180 M^{-1}, with stereoseletive differences in the formation constant for pairs of enantiomers of 11 % to 15 % [5]. Thus the chiral separation of LV and ME by CE is performed on the basis of a very weak interaction. This is possible because (i) CE provides high separation efficiency (e.g., in Figure 2, with 0.25 M γ-CD, theoretical plate numbers are in the range 240,000 to 350,000), and (ii) high concentrations of γ-CD may be added (up to 0.25 M in this case).

Effect of Temperature

Figure 6 shows electropherograms at different capillary oven temperatures, with a γ-CD concentration of 0.2 M. Increasing the temperature from 25 °C to 40 °C, the

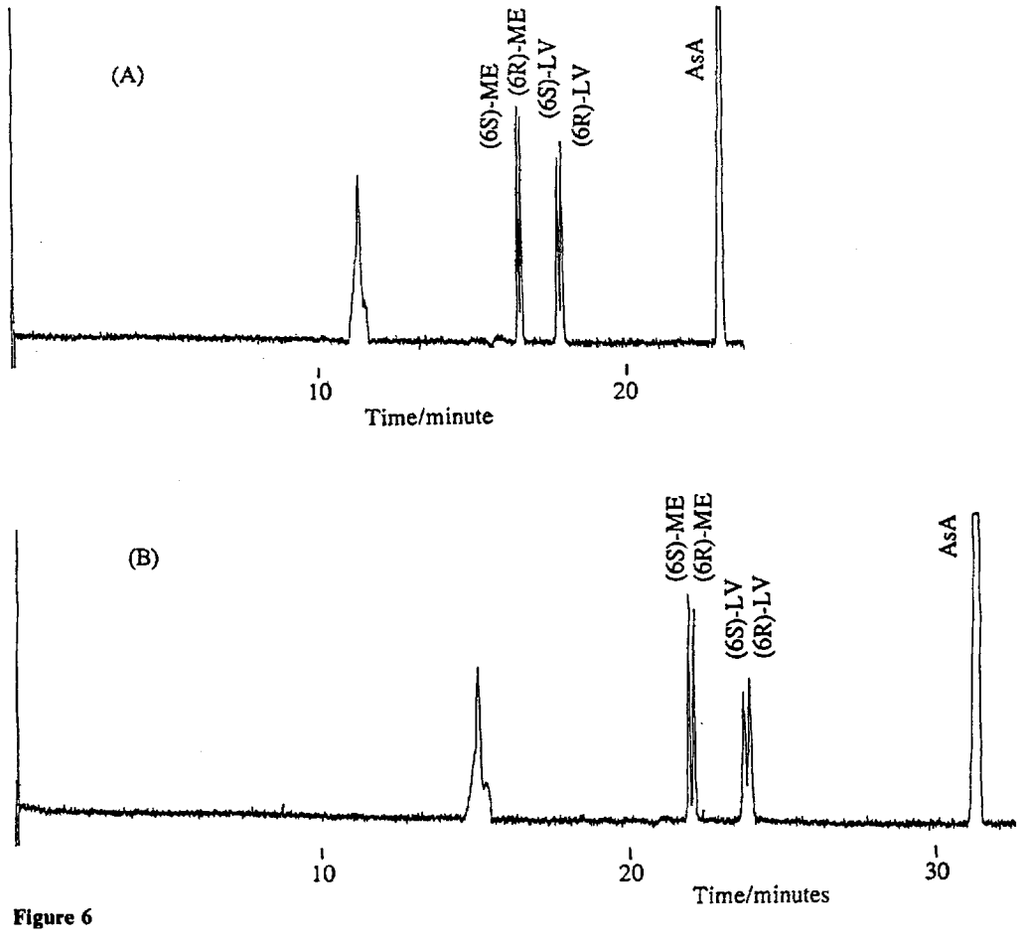

Figure 6

Effect of temperature on the separation of LV and ME. CE conditions: capillary, 72 cm × 51 μm i.d. (50 cm to detector); buffer, sodium phosphate (pH 7, I = 0.1) containing 0.2 M γ-CD and 6 M urea; voltage, 30 kV; temperature, (A) 40 °C, (B) 25 °C.

resolution (R) of the LV diastereoisomers increased from 0.93 to 1.07, while that of the ME diastereoisomers decreased from 1.18 to 1.08. This is at least in part due to a change in the separation efficiencies for LV and ME with temperature. The plate numbers for the LV diastereoisomers were 180,000 (6-S) and 200,000 (6-R) at 25 °C, and increased to 270,000 (6-S) and 360,000 (6-R) at 40° C. Conversely, the plate numbers for the ME diastereoisomers decreased from 470,000 (6-S) and 640,000 (6-R) to 420,000 (6-S) and 510,000 (6-R) on increasing the temperature from 25 C to 40 °C. There are velocity increases of 36 % (AsA), 33 % (ME) and 32 % (LV) on increasing the temperature from 25 °C to 40 °C, presumably reflecting a change in buffer viscosity. The velocity differences between the diastereoisomers also increase, by 18 % for ME and 22 % for LV. There may be a number of explanations for the differences seen between LV and ME. The effects of increasing temperature may be to: (i) decrease the stability of the complex, resulting in the loss of stereoselectivity; (ii) accelerate the kinetic processes of the complexation; (iii) possibly reduce adsorption onto the inner capillary wall; (iv) reduce the buffer viscosity, leading to increased diffusional broadening.

Effect of pH, Ionic Strength and Urea Concentration

Figure 7 shows the effect of pH and Figure 8 the effect of ionic strength of the buffer upon the migration velocities of the LV and ME diastereoisomers, and the velocity difference between the diastereoisomers. With increasing pH from 5 to 7, the electrophoretic velocity toward anodic end increased, based on the charge of the carboxyl groups of the tetrahydrofolates. Further increase in pH from 7 to 9 did not change the electrophoretic velocity. The velocity of the electroosmotic flow displayed a similar pH dependence. The velocity differences between the diastereoisomers were almost the same at pH 7 and pH 9, but decreased dramatically with decreasing pH from 7 to 5. This results in a change in chiral resolution (R) for both LV and ME from ≈ 0.5 at pH 5 to ≈ 1.1 at pH 7 or 9. The increased velocity difference and resolution at higher pH is thought to occur because increasing the charge on the solutes increases the difference in velocity between the free solute and the cyclodextrin-solute complex.

Changes in the ionic strength of the buffer caused relatively minor effects compared with changes in the buffer pH. With increasing ionic strength from 0.1 to 0.3, the migration times as well as the diastereomeric resolutions increased. The increase in migration times is due partly to a slight decrease in the electrophoretic velocity of the solutes (Figure 8), and also to a reduction in the electroosmotic flow velocity. On going

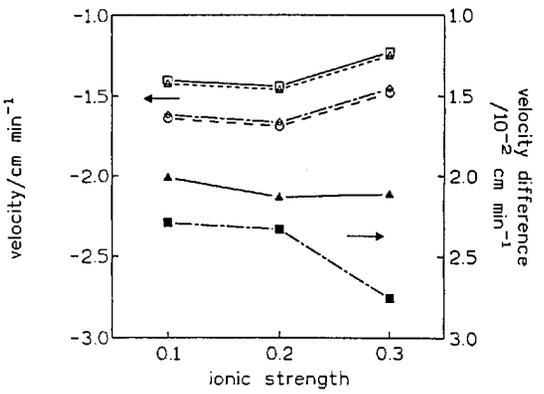

Figure 8

Effect of buffer ionic strength on the electrophoretic velocity of the diastereoisomers of R-LV (o), S-LV (◊), R-ME (Δ) and S-ME (□), and the velocity difference between the stereoisomers of LV (■) and ME (▲). CE conditions: capillary, 72 cm × 51 μm i.d. (50 cm to detector); buffer, sodium phosphate (pH = 9) containing 0.2 M γ-CD and 6 M urea; voltage, 30 kV; temperature, 40 °C.

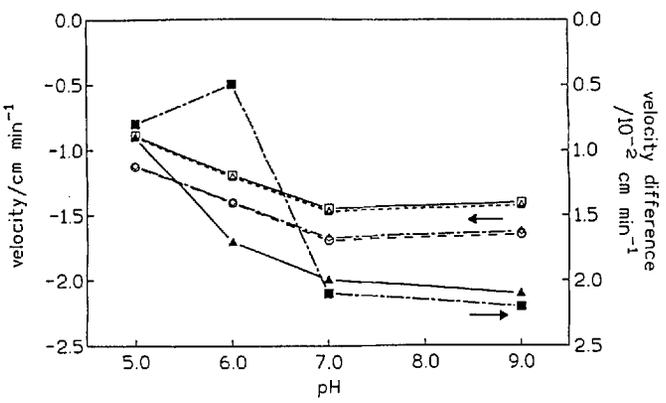

Figure 7

Effect of buffer pH on the electrophoretic velocity of the diastereoisomers of R-LV (o), S-LV (◊), R-ME (Δ) and S-ME (□), and the velocity difference between the stereoisomers of LV (■) and ME (▲). CE conditions: capillary, 72 cm × 51 μm i.d. (50 cm to detector); buffer, sodium phosphate (I = 0.1) containing 0.2 M γ-CD and 6 M urea; voltage, 30 kV; temperature, 40 °C.

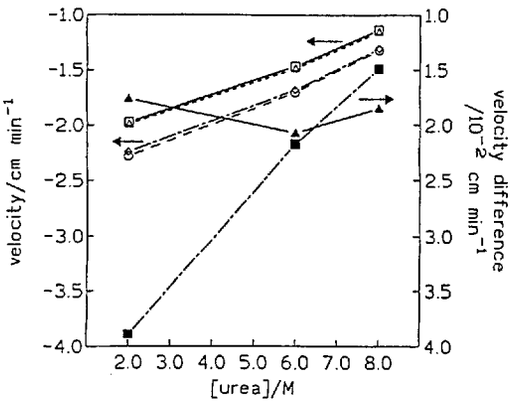

Figure 9

Effect of urea concentration on the electrophoretic velocity of the diastereoisomers of R-LV (o), S-LV (◊), R-ME (Δ) and S-ME (□), and the velocity difference between the stereoisomers of LV (■) and ME (▲). CE conditions: capillary, 72 cm × 51 μm i.d. (50 cm to detector); buffer, sodium phosphate (pH = 7) containing 0.2 M γ-CD and 2, 6 or 8 M urea; voltage, 30 kV; temperature, 40 °C.

from I = 0.1 to 0.3, R increased from 1.05 to 1.11 for LV, and from 1.10 to 1.18 for ME. This effect is in part due to a slight increase in separation efficiency, and for LV, a slight increase in velocity difference between the stereoisomers (Figure 8). The increase in separation efficiency may be explained since higher buffer salt concentrations are expected to provide reduced electromigration dispersion and improved peak stacking [29].

Figure 9 shows the effect of the urea concentration the velocities of LV and ME, and the differences in the velocities between the stereoisomers. The overall trend in the reduction in velocity of both LV and ME is similar. A similar reduction in electroosmotic flow occurs, indicating that the reduction in the velocity of the solutes may be attributed mostly to an increase in the solution viscosity upon addition of urea. However, while the velocity difference for ME changes only a little with increasing urea concentration, for LV there is a dramatic decrease in the velocity difference as the urea concentration is increased; this cannot be attributed to viscous effects, and must reflect changes in the strength of the complexes formed between the tetrahydrofolates and the CD. If the effects of viscosity (as measured by the reduction in electroosmotic flow) on the velocity difference are taken into account, the velocity difference between the ME diastereoisomers is approximately doubled on going from 2 M to 8 M urea, while the velocity difference between the LV stereosiomers is reduced by about 25 %. This is reflected by a large increase in R for ME which goes from 0.45 (2 M urea) to 1.08 (6 M urea) to 1.21 (8 M urea). There are also changes in N, with values of approximately 200,000 at 2 M urea, 425, 000 at 6 M urea and 320,000 at 8 M urea. For LV, R changes only slightly, the values being 0.89, 1.07 and 0.98 respectively. In this case, the reduction in mobility difference is in part compensated by an increase in efficiency, from N ≈ 150,000 with 2 M urea, 300,000 with 6 M urea and 270,000 with 8 M urea. It has been postulated above that LV may form an inclusion complex with γ-CD, while ME may bind to the exterior of the CD. The destabilisation by urea of the LV-CD but not the ME-CE complex supports the idea of different binding mechanisms for the two solutes.

Reversal of Elution Order

It is sometimes useful to reverse the elution order of optical isomers if there are tailing peaks and the separation does not achieve baseline resolution. In such cases when the concentrations of the optical isomers are considerably different, for example in case of the measurement of large enantiomeric excesses, it is desirable to elute the isomers with the lower concentration faster than its antipode. In HPLC using Pirkle-type stationary phases [30], the elution order can be reversed by changing the stationary phase to one modified with the ligand of opposite chirality. Otherwise, reversal of elution order is usually difficult.

Using CE with CD additives, the elution order of ionic stereoisomers can be reversed simply by adding a cationic surfactant such as CTAB into the separation buffer, as long as the stereoselectivity of the chiral selector is not effected by the presence of the surfactant. As shown by Altria and Simpson [22], addition of CTAB causes the direction of electroosmosis to be reversed, and a negative voltage must be applied at the injection end with the isomers detected at the anodic end. As long as the chiral discrimination is not significantly changed by adding the surfactant, the stereoisomer which originally had the highest effective mobility against the electroosmotic flow (and consequently had the slower overall velocity, and eluted second), moves fastest when the direction of electroosmosis is reversed, and thus elutes first. Figures 10 shows electropherograms using a pH 7 buffer containing both 25 mM CTAB and 0.24 M γ-CD with 5.4 M urea. The elution order was (6R)-LV < (6S)-LV < (6R)-ME < (6S)-ME, which is completely opposite from that observed before adding CTAB.

Application to the Analysis of Plasma Samples

To investigate the applicability of this method, LV and ME diastereoisomers spiked into samples of human plasma were analysed. As mentioned above, a higher γ-CD concentration is preferable from the view-point of resolution, but it results in a longer analysis time. Higher CD concentrations also give increased baseline noise. The effects of temperature, buffer pH and ionic strength and the urea concentration on the resolution of LV and ME must also be considered. Clean up of the plasma sample was achieved by a pretreatment consisting of denaturation of the plasma proteins by addition of urea [19, 20] to release the bound tetrahydrofolates and subsequent ultrafiltration to remove macromolecules. Because of the high salt content of the ultrafiltered plasma samples, operation with a high ionic-strength buffer was preferred, to give some degree of peak-stacking. The optimum separation electrolyte composition was selected as a sodium phosphate solution (pH 9, ionic strength = 0.3) containing 0.2 M γ-CD and 6 M urea. The temperatures was 40° C and the

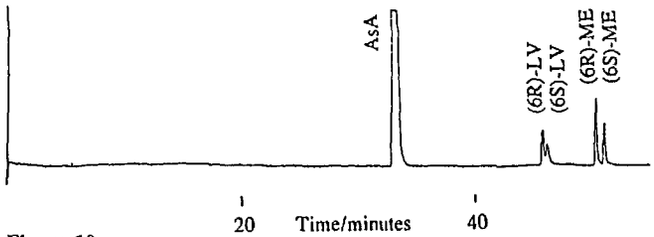

Figure 10

Separation of LV and ME using buffer containing γ-CD and CTAB. CE conditions: capillary, 72 cm × 51 μm i.d. (50 cm to detector); buffer, sodium phosphate (pH 7, I = 0.1) containing 0.24 M γ-CD, 5.4 M urea and 25 mM CTAB; voltage, - 20 kV; temperature, 25 °C. Sample concentration, 58 μM (6R)-LV, 34 μM (6S)-LV, 102 μM (6R)-ME and 45 μM (6S)-ME.

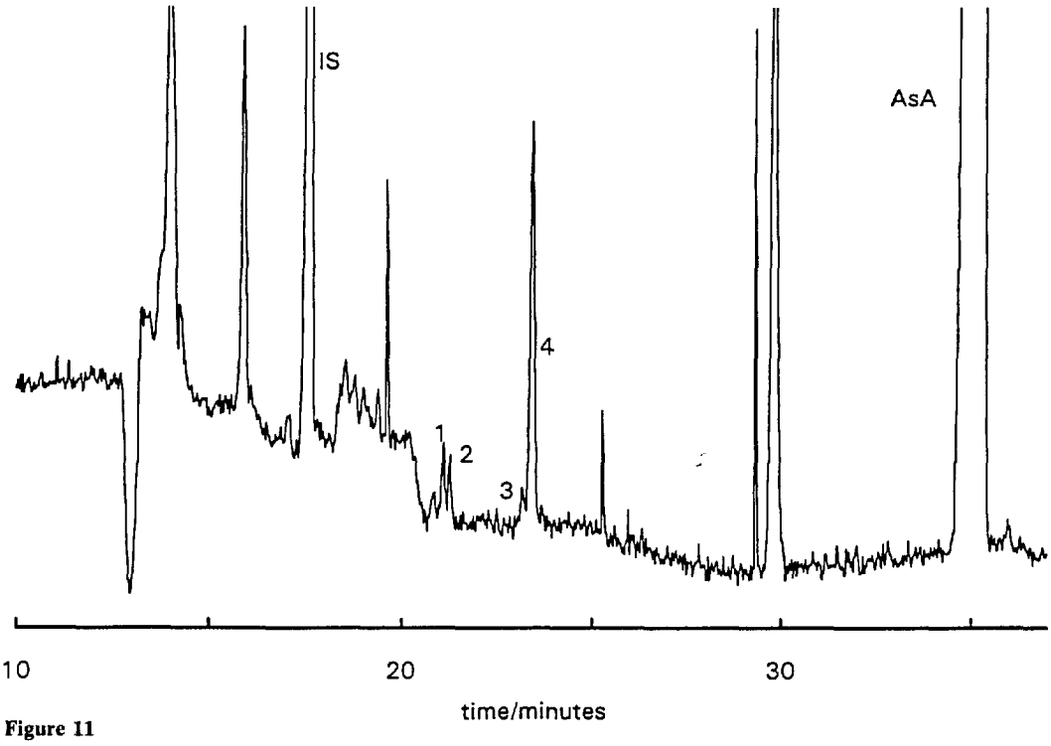

Figure 11

Electropherogram of spiked plasma sample. CE conditions: capillary, 72 cm × 51 μm i.d. (50 cm to the detector); buffer, sodium phosphate (pH 9, I = 0.3) containing 0.2 M γ-CD and 6 M urea; voltage; 30 kV; temperature, 40 °C; injection, by vacuum 20.0″ Hg for 5.0 s. Marked peaks are (1) 5.7 μM 6S-ME; (2) 5.0 μM R-ME; (3) 3.25 μM S-LV; (4) 38.2 μM R-LV; IS – internal standard.

applied voltage was 30 kV. Figure 11 shows an electropherogram of a spiked plasma sample containing 38.2 μM (6R)-LV, 3.25 μM (6S)-LV, 5.00 μM (6R)-ME and 5.70 μM (6S)-ME. The concentrations of (6R)-LV, (6S)-LV and (6S)-ME are the same as the steady-state concentrations found in a patient treated with a continuous i.v. infusion of (R,S)-LV [31]. Although it is believed that *in vivo* (6R)-LV is not metabolized into (6R)-ME [32], (6R)-ME was also added to demonstrate that chiral separation is achieved. There were no interferences by plasma components with the peaks of the tetrahydrofolates or the internal standard. The separation shown in Figure 11 clearly indicates the potential of the present system for the analysis of LV and its major metabolite in plasma. However, further improvements in sensitivity will be necessary before this is a clinically useful method for monitoring of LV and ME. A suitable extraction step giving both sample desalting and pre-concentration of the analytes may be all that is necessary to achieve the required sensitivity.

Conclusions

The simultaneous separation of LV and ME diastereoisomers can be achieved by CE with g-CD as a chiral selector. CE separations were carried out using a small volume of buffer solution containing the expensive g-CD additive. Although the interactions between g-CD and the tetrahydrofolates are weak, the high efficiency of CE and the high g-CD concentration in the buffer compensate for this. It is possible to estimate the formation constant and the velocity for the solute-CD complexes, even with such weak interactions. Care must be taken in correcting for viscosity effects of the chiral selector at high concentrations (direct viscosity measurements are not suitable for making such corrections), and the precision in estimation of binding constants and the CD-solute complexes is rather low. There is evidence that the chiral selection mechanism for LV and ME may be different: (i) The diastereoisomers of ME are separated using a-, b- and g-CD, but only g-CD separates the diastereoisomers of LV; (ii). Changes in temperature as well as urea concentration lead to opposite effects on the diastereomeric resolution of LV and that of ME. The effect of the different sizes of CD suggests that the binding of ME does not involve inclusion into the CD, while that of LV does. There is a relatively large difference in the estimated values for v'_{epCS} for LV but not for ME; however, the precision of these estimates is not adequate to be confident of a difference. It was shown that using CE with g-additives the diastereoisomers of LV and ME may be separated in human plasma. In addition, it was demonstrated that the elution order of the LV and ME diastereoisomers could be reversed. This was achieved by adding a cationic surfactant and reversing the polarity of the separation voltage; this should also be possible with other ionic stereoisomers.

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