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Enantiomeric separation of the novel growth hormone secretagogue MK-0677 by capillary zone electrophoresis

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Abstract

MK-0677 {(*R*)-2-amino-N-[2-(1,2-dihydro-1-(methylsulfonyl) [3H-indole-3,4′-piperidin]-1′yl]-2-oxo-1-[(phenylmethoxy)-methyl]ethyl-2-methylpropanamide-monomethane sulfonate} is a novel orally-active growth hormone secretagogue. The *R*- and *S*-enantiomers were separated by capillary zone electrophoresis (CZE) using β-cyclodextrin (β-CD) as the chiral selector in a phosphate buffer containing L-tartaric acid and ethanol. Resolution of the enantiomers requires optimizing the buffer constituent concentrations, apparent pH (pH_{app}) and temperature, *T*. The presence of an ion-pairing reagent such as tartaric acid is essential to achieve separation. The log of the separation factor, ln α, increases linearly with $1/T$ over the range from 10°C to 45°C. It is shown that the equilibrium 1:1 CD–enantiomer complex model of Wren and Rowe is a limiting case of the multiple equilibria model of Rawjee and Vigh. An analytical method for MK-0677 based on the CZE separation of it from its *S*-enantiomeric impurity was validated in terms of limit of detection, limit of quantitation, UV detector linearity, precision, accuracy and ruggedness. The best working conditions include a background electrolyte containing 40 m*M* L-tartaric acid, 24 m*M* NaH_2PO_4, 30 m*M* β-CD and 25% (v/v) ethanol at pH_{app} 4.2; a UV detector at 200 nm; and a 52 cm effective length×76 μm I.D. fused-silica capillary operated at 25°C.

Keywords: Enantiomer separation; Growth hormone secretagogue; MK-0677

1. Introduction

Capillary zone electrophoresis (CZE) and high-performance liquid chromatography (HPLC) are the most powerful analytical techniques for the separation of enantiomers. Compared with HPLC, CZE offers higher separation efficiency, easier exchange of separation media, smaller sample volumes and quantities of reagents and lower cost. Addition of chiral selectors to the CZE background electrolyte (BGE) allows formation of transient diastereomeric complexes with the enantiomers. If the complex formation rate is fast relative to the electrophoretic migration rate, and if the different conformations of the two enantiomers result in different complex stabilities, the electrophoretic mobilities of the two may differ. Various chiral selectors have been used in CZE, as recently reviewed [1]. However, the cyclodextrins (CDs) and their derivatives have become the most popular. Rawjee and Vigh [2] have recently summarized this extensive literature. Since CDs are neutral compounds, only ionic enantiomers can be separated by CZE with these selectors.

Interest in growth hormone secretagogues has

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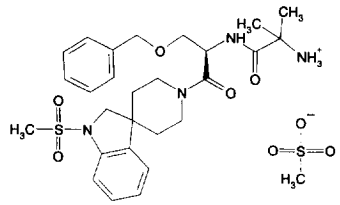

Fig. 1. Structure of MK-0677.

intensified during the past several years. MK-0677, whose structure is shown in Fig. 1, belongs to a new structural class of orally-active growth hormone secretagogues [3]. Pre-clinical and clinical studies have shown the safety and efficacy of MK-0677, and indicate it will satisfy important medical needs [4]. The biologically-active form is the *R*-isomer. Thus the *S*-isomer is considered an enantiomeric impurity, which requires development of an analytical method for the separation and determination of the two enantiomers with accurate detection of the *S*-isomer at levels <0.5%.

Initially a HPLC method with an ovomucoid (OVM) chiral recognition protein column was used to achieve the chiral separation. However, OVM columns are quite expensive and have a lifetime much shorter than expected for this application. For these reasons we developed the CZE procedure described here. The CZE method is superior in terms of detection limits and tailing factors. Fig. 2 compares a HPLC chromatogram and a CZE electropherogram of a racemic mixture of MK-0677, which illustrates the difference between the results of the two techniques.

Two models of CZE enantiomeric separations using CDs have been put forth. The first, developed by Wren and Rowe [5–7], is based on the formation of 1:1 equilibrium complexes of the enantiomers with the chiral selector. From this model they showed there is an optimum CD concentration for maximizing resolution. Their model was extended by Penn and coworkers [8–13], who showed how to derive binding constants of the CD–enantiomer complexes from CZE measurements. The second, a more elaborate and more general model introduced by Rawjee and coworkers [2,14–17], is based on competing multiple equilibria, taking explicitly into account the effects on mobility, selectivity and

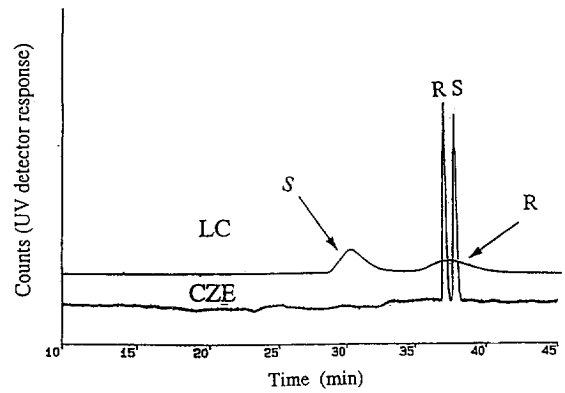

Fig. 2. Side-by-side comparison of HPLC and CZE separation of MK-0677 enantiomers. HPLC conditions: 150×4.6 mm I.D. Ultron-ES OVM protein column; eluent, 15 m*M* ammonium phosphate–acetonitrile (83:17, v/v), 1.0 ml/min; 27°C; UV detector, 210 nm. CZE conditions: fused-silica capillary, 52 cm effective length (72 cm total length)×76 μm I.D.; BGE, 24 m*M* NaH_2PO_4, 40 m*M* L-tartaric acid, 30 m*M* β-CD, 25% (v/v) ethanol, pH_{app} 4.2; 25±1°C; UV detector, 200 nm; applied voltage, 20 kV.

resolution, of the enantiomer p*K* values and the formation constants of the complexes of both the dissociated and undissociated enantiomers with the CDs, the pH and the CD concentration. In this work we point out the first model is a limiting case of that of Rawjee and coworkers. Factors were studied that govern the enantiomeric separation, i.e., the concentrations of β-CD, L-tartaric acid (an ion-pairing reagent), ethanol and NaH_2PO_4; the apparent pH (pH_{app}) of the buffer; and the temperature and applied voltage. In addition, to meet the current Good Manufacturing Practices (cGMP) guidelines [18,19], the CZE method developed was validated in terms of precision, accuracy, ruggedness, linearity, limit of detection and limit of quantitation. The method was applied to the analysis of the bulk drug manufactured for pre-clinical and clinical studies.

2. Experimental

2.1. Instrumentation

Both an Applied Biosystems ABI 270A and a Hewlett-Packard HP^{3D} CE were used. The 76 μm I.D. fused-silica capillary in the ABI instrument had

an effective length of 52 cm (total length, 72 cm) (Polymicro Technologies, Phoenix, AZ, USA) and was operated at 25±1°C. In the Hewlett-Packard CE the capillary was 56 cm effective length (63 cm total)×75 μm I.D. (Wilmington, DE, USA), maintained at 25±0.1°C. Both the UV detector in the ABI and the diode array detector in the Hewlett-Packard instrument were operated at 200 nm. In both, hydrodynamic sample injection was applied for 2 s at 50 mbar pressure, and the applied voltage was 20 kV. Data collection and handling were carried out with the PE Nelson Access Chrom System (Cupertino, CA, USA).

For HPLC, a Spectra-Physics instrument was used with an Ultron-ES OVM protein column (150×4.6 mm I.D.; Mac-Mod Analytical, Chadds Ford, PA, USA), with 1.0 ml/min of 15 m*M* ammonium phosphate–acetonitrile (83:17, v/v) mobile phase at 27°C and a UV detector at 210 nm. To reduce the baseline noise caused by the presence of acetonitrile in the eluent, it was necessary to operate the HPLC detector at a longer wavelength than with the CE, as indicated in the caption for Fig. 2.

2.2. Reagents

MK-0677 and its *S*-enantiomer were prepared by the Process Research Department of Merck Research Laboratories (Rahway, NJ, USA). L-Tartaric acid and mesityl oxide were obtained from Aldrich (Milwaukee, WI, USA), β-CD and all the ion-pairing reagents from Sigma (St. Louis, MO, USA) and NaH_2PO_4, methanol (MeOH), ethanol (EtOH), 2-propanol (IPA) and 50% NaOH solution from Fisher Scientific (Springfield, NJ, USA). Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA).

2.3. Solutions

Except when systematically varying the composition, the background electrolyte (BGE) used was 40 m*M* L-tartaric acid (0.60 g/100 ml), 24 m*M* NaH_2PO_4 (0.34 g/100 ml) and 30 m*M* β-CD (3.4 g/100 ml) in ethanol–water (25:75), adjusted with 50% NaOH to an apparent pH, pH_{app}, of 4.2 (the pH value is apparent because the pH meter was standardized with aqueous buffers). The BGE was first prepared without the β-CD, which was dissolved in that solution with stirring and warming to 60°C. The EtOH solubilizes the β-CD beyond its aqueous saturation value of about 15 m*M* [14]. A test solution of a racemic mixture of the enantiomers (0.08 m*M* each, 5 mg each/100 ml) was prepared in BGE without β-CD. Eight linearity test solutions containing MK-0677 in BGE without β-CD were prepared over the range from 0.32 μg/ml to 0.54 mg/ml (i.e., 0.08% to 135% of the target concentration of 0.4 mg/ml). The recovery test solution contained 0.4 mg/ml MK-0677 spiked with 1.4 μg/ml (0.35%) of the *S*-isomer in BGE without β-CD. The neutral marker of electroosmotic flow was mesityl oxide, injected as a 0.3 m*M* solution (0.03 mg/ml) in BGE. All solutions were filtered through a 0.45 μm Nylon-66 membrane syringeless filter (Whatman, Clifton, NJ, USA).

2.4. Capillary preparation

New capillaries were flushed with 1 *M* NaOH for 30 min followed by deionized water for 10 min and 0.2 *M* NaOH for 60 min. Between injections, the capillary was rinsed with 0.2 *M* NaOH for 3 min followed by BGE for 3 min.

2.5. Viscosity correction

The analyte effective mobilities were determined in the usual way from the difference in migration times of the analytes and the mesityl oxide. Correction of the mobilities for change in buffer viscosity, η, with changing concentration of β-CD was made by measuring the current, i, which all else constant is proportional to the buffer viscosity [13]. The electrically neutral CD has no effect on the current per se. Since $\eta/\eta_0 = i/i_0$, where the subscript 0 refers to the buffer with no CD,

$$\mu_{corr} = \mu_{meas} i/i_0 \quad (1)$$

3. Results and discussion

3.1. Effect of β-CD concentration

The concentration of β-CD, [β-CD], was varied

from 0 to 50 m*M*. As shown in Fig. 3a, there is no difference in effective mobilities, μ_{eff}, of the two enantiomers with no chiral selector present. As [β-CD] was increased, μ_{eff} (viscosity-corrected) decreased because of the larger size of the inclusion complex and the increasing fraction of analyte complexed. The EOF was essentially independent of, or decreased slightly, with [β-CD] (Fig. 3b). The $\Delta\mu = (\mu_R - \mu_S)$ is seen to pass through a maximum at [β-CD] = 30 m*M*.

Rawjee et al. [15] showed that the effective mobility, μ, of a weak base enantiomer at [β-CD] = *C*, can be expressed (using the symbolism of Penn et al. [9])

$$\mu = \mu_0 \frac{1 + \frac{\mu_\infty}{\mu_0} KC}{1 + KC + \frac{[OH^-]}{K_b}(1 + K_u C)} \quad (2)$$

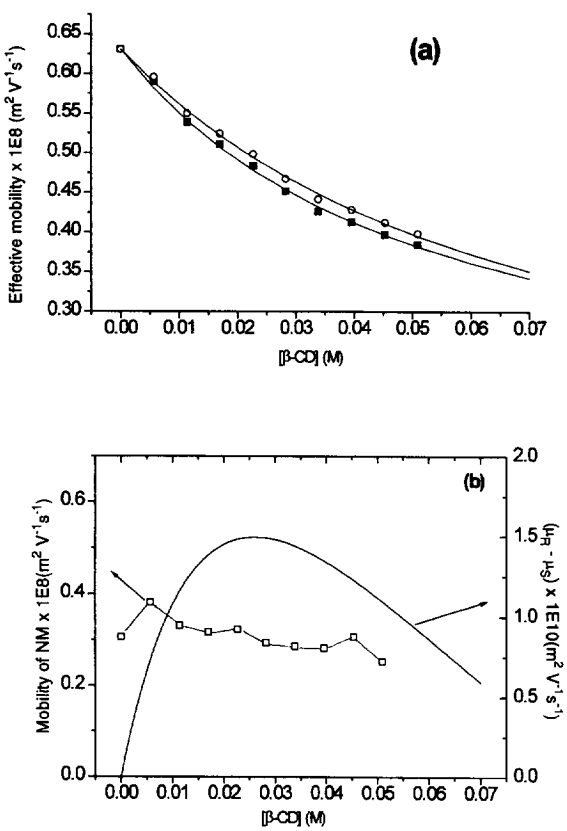

Fig. 3. Effect of [β-CD] on (a) enantiomer effective mobilities and (b) $\Delta\mu$ and electroosmotic flow. In (a), ■ = *S*-isomer, ○ = *R*-isomer. CZE conditions: as in Fig. 2 except [β-CD] was varied.

where μ_0 is the mobility of the uncomplexed compound (measured at $C=0$), μ_∞ is that of the completely complexed enantiomer, K is the binding constant of the dissociated form of the base, K_b is base dissociation constant and K_u is the binding constant of the undissociated form of the enantiomer with the β-CD. Where the third term in the denominator is small compared to $(1+KC)$, it is easily shown that

$$KC = \frac{\mu_0 - \mu}{\mu - \mu_\infty} \quad (3)$$

which is the equation used [8] in the rearranged form, Eq. (4), to which the experimental mobility data were fitted to obtain K values:

$$\mu = \frac{\mu_0 - \mu_\infty}{1 + KC} + \mu_\infty \quad (4)$$

The Wren and Rowe [5–7] model of 1:1 chiral selector–enantiomer equilibrium interactions as extended by Penn and coworkers [8–13] is thus seen to be a limiting case of the more elegant model of Rawjee and coworkers [2,14–17]. This limit would hold for low pH buffers. In our case, the $[OH^-]$ was $1.6 \cdot 10^{-10}$ and $K_b = 6.3 \cdot 10^{-7}$ and for any reasonable value of K_u, the third term in the denominator of Eq. (1) is negligible compared to $(1+KC)$. In Fig. 3a the points are the experimental net mobilities and the line is the best fit of the data to Eq. (4). A nonlinear least squares program written in C++ combined with a SAS statistical software package [18] were used to calculate the binding constants and μ_∞. From the fit, $K_{MK\text{-}0677} = K_R = 14.2 \pm 1.2\ M^{-1}$ and $K_{S\text{-isomer}} = K_S = 19.4 \pm 1.6\ M^{-1}$; $\mu_{\infty,R} = 0.13 \pm 0.04 \cdot 10^{-8}\ m^2\ V^{-1}\ s^{-1}$ and $\mu_{\infty,S} = 0.07 \pm 0.08 \cdot 10^{-8}\ m^2\ V^{-1}\ s^{-1}$. The experimental value of μ_0 is $0.063 \cdot 10^{-8}\ m^2\ V^{-1}\ s^{-1}$. The uncertainties (standard error) in the K values are less than 10%. These K values are rather smaller than those for example for tioconozole [8] or homatropine [15] and probably reflect both the presence here of ethanol in the BGE, which solubilizes the analytes and competes with them for interaction with the β-CD [6], and the fact that MK-0677 and its isomer are much larger molecules which cannot fit as easily inside the cavity of the β-CD.

In the experience of Penn and coworkers [10,13], values of μ_∞ for the two enantiomers have always been observed to be identical, although there is no a

priori reason why this should always (or even ever) be true. When this is the case, the difference in enantiomer mobilities, $\Delta\mu$ can be shown [5] from Eq. (4) to be

$$\Delta\mu = \frac{C(\mu_0 - \mu_\infty)(K_S - K_R)}{1 + C(K_S + K_R) + K_S K_R C^2} \quad (5)$$

from which it can be shown by differentiation there is an optimum C, C_{opt}, resulting in $\Delta\mu_{max}$,

$$C_{opt} = (K_R K_S)^{-1/2} \quad (6)$$

For our derived binding constants, Eq. (6) gives C_{opt}=60 mM β-CD. However, the $\Delta\mu/C$ curve based on the experimental values obtained from Fig. 3a maximizes at about 30 mM β-CD, as shown in Fig. 3b. In our case there is probably no significant difference in the μ_∞ values, given the relatively large uncertainties in the values. The reason for the discrepancy between the observed and theoretical C_{opt} values is probably caused [13] by the fact that although the curves in Fig. 3a appear to be converging, the β-CD solubility limit precludes a full experimental definition of the curves.

The enantiomers studied here are weak bases, but the same comparison of the Wren–Rowe and Rawjee and coworkers models should hold for weak acids at high pH values.

3.2. Effect of alcohol concentration

Ethanol was added to the BGE to help solubilize the β-CD, but it also affected the separation. As shown in Fig. 4a,b, the effective mobilities of the enantiomers and the EOF decreased somewhat with increasing concentration of ethanol over the range from 10 to 30% (v/v). Increasing the ethanol concentration reduces the K_R and K_S values by increasing the solubility of both enantiomers in the BGE and perhaps through competitive interaction for the β-CD. EOF is suppressed because the ethanol reduces the ζ-potential on the silica wall surface [19], and also increases the viscosity of the BGE.

The enantiomeric resolution, R_s, however, increased with ethanol concentration by a factor of approximately 1.8, although the $\Delta\mu$ remained essentially constant. For a CZE system in which longitudinal diffusion is the sole mechanism of peak

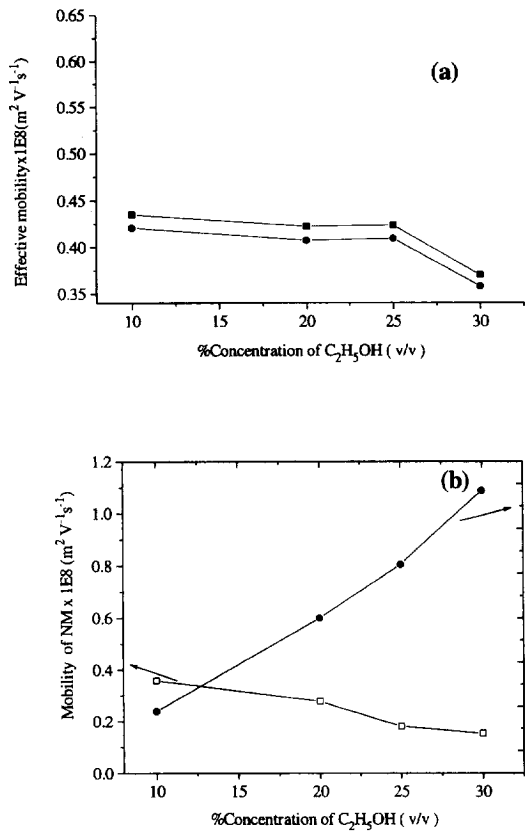

Fig. 4. Effect of ethanol concentration on (a) enantiomer effective mobilities and (b) resolution and electroosmotic flow. In (a), ■=R-isomer, ●=S-isomer. CZE conditions: same as in Fig. 2 except [EtOH] was varied.

spreading (which may be a rare case [20]), it can be shown [21] that

$$R_s = (\Delta\mu/5.66)\left[Vl/LD_{avg}(\mu_{avg} + \mu_{eof})\right]^{1/2} \quad (7)$$

where V is the applied voltage, l is the effective capillary length, L is the total length, D_{avg} is the mean diffusion coefficient of the enantiomers and μ_{avg} is their average mobility. Over the range of ethanol concentrations studied, the decreases in μ_{avg}, μ_{eof} and D_{avg} (through increase in η) can account quantitatively for most of the increase in R_s.

To substantiate the cause of the effect of the organic modifier, additional experiments were carried out using methanol (MeOH) and 2-propanol (IPA), both at the same 25% (v/v) concentration as the ethanol. The concentration of β-CD in the BGE was varied in order to determine the binding con-

Table 1
Effect of alcohol on enantiomer: β-CD binding constants

Alcohol	K_R (M^{-1})	K_S (M^{-1})
Methanol	184±11	205±12
Ethanol	97.9±8.0	102±10
2-Propanol	19.3±1.2	29.1±2.1

stants from the nonlinear least squares fit of the data to Eq. (4). The results are given in Table 1. As might be expected for molecules with large hydrophobic regions, 2-propanol should be a better solvent than ethanol than methanol. Similarly, 2-propanol should interact with the hydrophobic interior of the β-CD to a greater extent than ethanol than methanol. Thus the binding constants would be expected to be in the order $K_{MeOH} > K_{EtOH} > K_{IPA}$, which is just what is found.

3.3. Effect of temperature

The electropherograms in Fig. 5 illustrate the improvement in separation resulting from decreasing the temperature from 45°C to 10°C, but at the expense of run time. Ambient temperature (25°C) seems a good compromise in this case. Quantitatively, the increase in the separation factor [22,23],

$$\alpha = (\mu_S - \mu_{eof})/(\mu_R - \mu_{eof}) \quad (8)$$

with decreasing temperature is shown in Fig. 6 as ln α vs. $1/T$. The linearity of the plot ($r^2 = 0.996$) reflects the constancy over this temperature range of the interaction of the enantiomers with the β-CD.

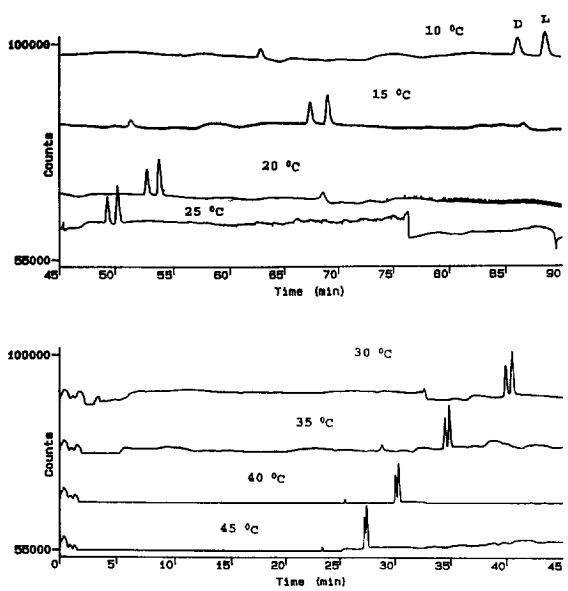

Fig. 5. Electropherograms of MK-0677 enantiomers at different temperatures. CZE conditions: HP^{3D} system; fused-silica capillary, 56 cm effective length (63 cm total length)×75 μm I.D.; BGE, as in Fig. 2; 25±0.1°C; UV detector, 200 nm; applied voltage, 20 kV.

3.4. Effect of pH_{app}

Rawjee and coworkers [2,14–17] have extensively demonstrated the importance of both pH and CD concentration in the separation of weakly acidic or basic enantiomers. This is also clear in this work, as illustrated in Fig. 7. The pH_{app} was varied from 2.5 to 8.0 by drop-wise addition of 50% NaOH solution to the BGE. The mobility of the neutral marker of EOF is essentially a titration curve of the silanol groups on the fused-silica capillary wall. The enantiomer effective mobilities decrease as the pH_{app} is increased from pH 2.5 to 6. This decrease can be attributed in part to the increase in ionic strength of the BGE from the Na^+ ions [24,25]; the BGE was not "Na^+-balanced" [17]. In addition, as the pH_{app} approaches the pK_a of the enantiomers, they begin to lose their positive charge and their mobility drops. Binding constants for the enantiomer–β-CD complexes were measured as above by varying the [β-CD], all else in the BGE held constant, at pH_{app} 4.2 and 7.0. The results are given in Table 2. The K

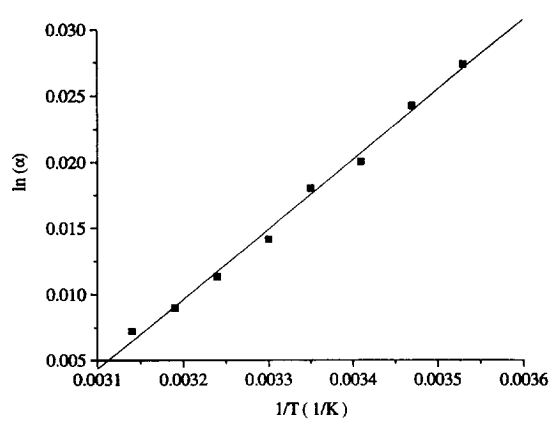

Fig. 6. Effect of temperature on separation factor. CZE conditions: as in Fig. 5 except temperature was varied.

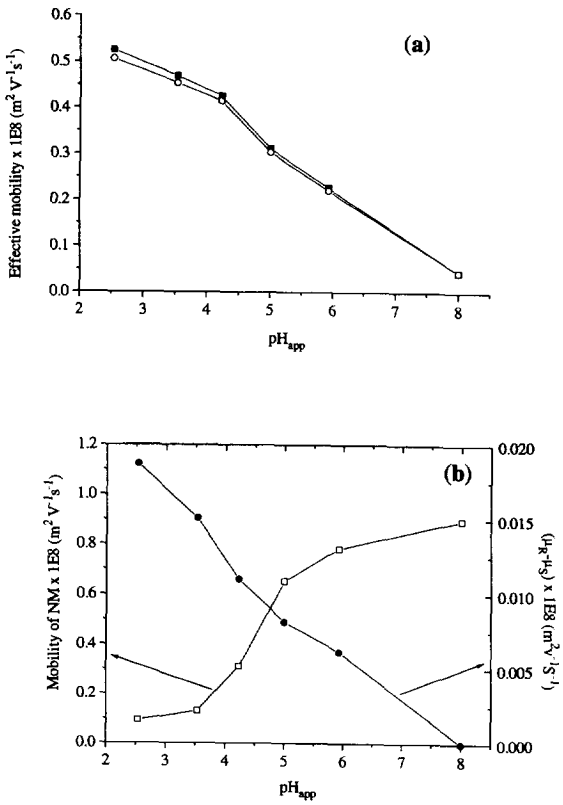

Fig. 7. Effect of pH_{app} on (a) enantiomer effective mobilities and (b) $\Delta\mu$ and electroosmotic flow. In (a), ■ = *R*-isomer, ○ = *S*-isomer. CZE conditions: as in Fig. 2 except pH_{app} was varied.

values are substantially lower at the higher pH_{app}, although the K_S/K_R ratio remains constant.

The resolution follows a trend with pH_{app} similar to the mobility, despite the reduced electromigration dispersion at the higher ionic strength. Decreasing resolution with increasing pH_{app} is associated with the increase in EOF and the loss of charge causing a reduction in binding constants, decreased mobilities, and smaller $\Delta\mu$. It cannot be determined based on our data whether MK-0677 should be categorized as a so-called type II or type III system in Rawjee et al.'s [14] scheme, i.e., whether only the dissociated enantiomers complex selectively with the β-CD, or both the dissociated and nondissociated forms do, respectively. For our purposes, the distinction is not very important since we have achieved a good separation with a reasonable analysis time.

Table 2
Effect of pH_{app} on enantiomer binding constants

pH_{app}	K_R (M^{-1})	K_S (M^{-1})
4.2	15.6±1.2	18.1±1.5
7.0	9.97±0.7	11.7±1.1

3.5. Effect of sodium phosphate concentration and ion-pairing reagent

As shown in Fig. 8, addition of NaH_2PO_4 up to 50 m*M* produced no change in $\Delta\mu$ and small changes in the effective mobility and EOF, but improved resolution through decreased electromigration dispersion of the peaks [24,25].

The concentration of L-tartaric acid (LTA), the ion-pairing reagent (IP) principally studied, was

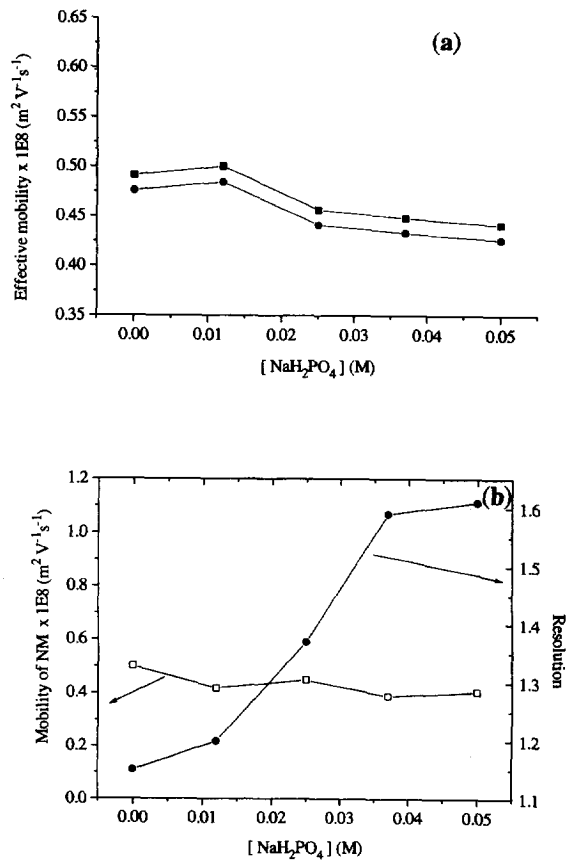

Fig. 8. Effect of sodium phosphate concentration on (a) enantiomer effective mobilities and (b) resolution and electroosmotic flow. In (a), ■ = *R*-isomer, ● = *S*-isomer. CZE conditions: as in Fig. 2 except sodium phosphate concentration was varied.

varied from 0–60 m*M*, all else in the BGE held constant. The initial addition of LTA to give a concentration of 20 m*M* caused a large decrease in the effective mobility and an increase in the resolution, as shown in Fig. 9. Further increase in the LTA concentration to 60 m*M* produced smaller changes. We believe these changes result from two effects of the LTA. First, at the pH_{app} used, 4.2, the negatively charged LTA can act as an IP with the positively charged amine group on the enantiomer. This causes a decrease in effective mobility by both reducing the net positive charge and increasing the hydrodynamic size of the enantiomer–β-CD complexes. Second, LTA can bind competitively with the β-CD, reducing the binding constants of the enantiomer complexes. The increase in resolution can be rationalized in terms of Eq. (7); the average mobility is decreased by the LTA, and the larger size of the ion-pair diminishes the mean diffusion coefficient.

Substitution of D-tartaric acid for LTA produced no change in the results, which suggests the LTA plays no role as a chiral recognition reagent in the enantiomeric separation.

Investigation of the effect of charge on the IPs was conducted by adding in place of LTA to the same BGE, 40 m*M* of citric acid (CIT), camphosulfonic acid (CSA), or 3-(N-morpholino)-propanesulfonic acid (MOPS). Fig. 10 shows electropherograms for each IP. The order of migration times is $t_{MOPS} < t_{CSA} < t_{LTA} < t_{CIT}$, which is the same order of increasing negative charge on the IPs. MOPS like CSA is a monoprotic acid, but protonation of the morpholino-N at pH 4.2 reduces its net negative charge. LTA is diprotic and CIT triprotic; at the pH used, neither is fully dissociated but CIT should be dissociated to a slightly greater extent. Higher negative charge should yield stronger ion-pairing, resulting in smaller binding constants and slower mobilities, as observed. Competitive binding of the IP for interaction with the CD cavity was studied by replacing the LTA in the BGE (containing 30 m*M* Na_2PO_4 rather than the usual 24 m*M*) sequentially with 40 m*M* of each of the homologous series of *n*-alkyl-sulfonic acids ranging from C_3 to C_{10} (excepting the C_9). These have the same charge but increasing ability to interact with the β-CD. Fig. 11 shows the electropherograms of the enantiomers. Migration time increases and resolution decreases with increasing sulfonic acid carbon number; no separation at all is

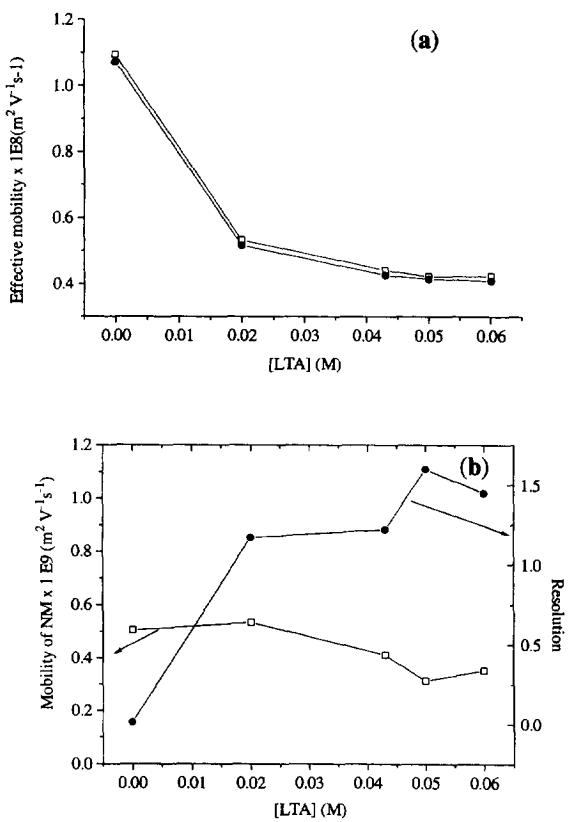

Fig. 9. Effect of L-tartaric acid concentration on (a) enantiomer effective mobilities and (b) resolution and electroosmotic flow. In (a), □=*R*-isomer, ●=*S*-isomer. CZE conditions: as in Fig. 2 except tartaric acid concentration was varied.

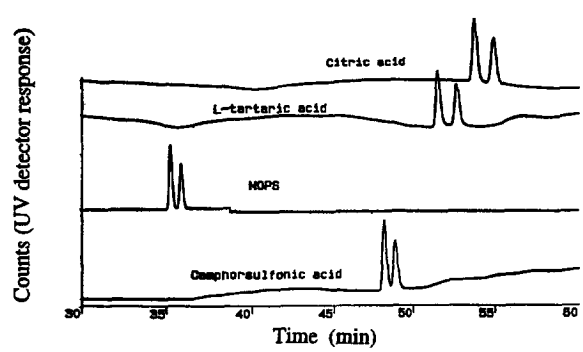

Fig. 10. Electropherograms of MK-0677 enantiomers using different ion-pairing reagents. CZE conditions: as in Fig. 5 except ion-pairing reagent was varied; concentration of reagent in each case, 40 m*M*.

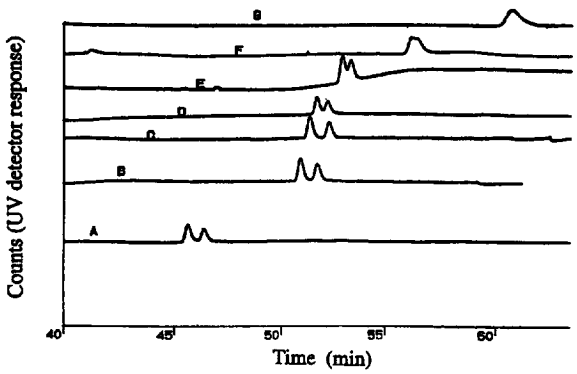

Fig. 11. Electropherograms of MK-0677 enantiomers using different ion-pairing reagents. (A) Propanesulfonic acid; (B) butanesulfonic acid; (C) pentanesulfonic acid; (D) hexanesulfonic acid; (E) heptanesulfonic acid; (F) octanesulfonic acid; (G) decanesulfonic acid. CZE conditions: as in Fig. 5 except ion-pairing reagent was varied; concentration of sulfonic acid reagent in each case, 40 m*M*.

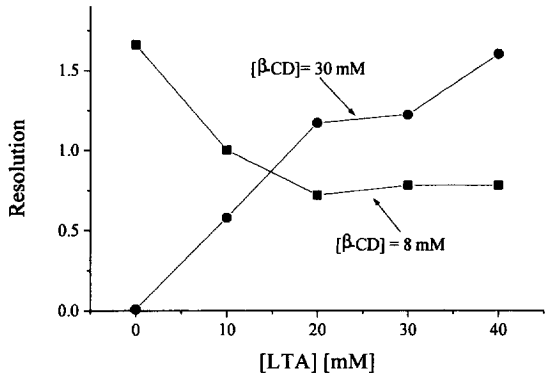

Fig. 12. Effect of L-tartaric acid concentration on resolution at two concentrations of β-CD. CZE conditions: as in Fig. 5 except concentrations of LTA and β-CD were varied.

observed for the *n*-decanesulfonic acid. This is reflected in the binding constants of the enantiomer–β-CD complexes in the same BGE with and without an ion-pairing reagent, as shown in Table 3 (BTSA is butanesulfonic acid). (Note that because of the slightly higher phosphate concentration, the *K* values in Table 3 for LTA as the IP differ slightly from those given above).

Wren and Rowe showed [6] that if the [β-CD] is larger than the optimum value in the organic modifier-free BGE, addition of an organic modifier which competes with the analytes for the chiral selector should increase the resolution; if less than the optimum value, the modifier should decrease R_s. The same should apply to IPs that compete with the analytes for the interaction sites in the β-CD. Table 3 gives the calculated C_{opt} for LTA as 10 m*M*. Resolution was measured for increasing LTA concentration, [LTA], in the presence of β-CD above (30 m*M*) and below (8 m*M*) C_{opt}. The results presented in Fig. 12 are entirely consistent with this prediction.

Table 3
Effect of ion-pairing reagent on enantiomer binding constants[a]

Ion-pairing reagent	K_R (M^{-1})	K_S (M^{-1})	C_{opt} (m*M*)
LTA	15.6±1.2	18.1±1.5	59.4
MOPS	48.1±3.5	52.2±4.5	20.0
BTSA	47.0±3.2	50.4±4.2	20.5
No ion-pairing reagent	97.9±8.0	102±10	10.0

[a] *K* values at 25°C. See Section 3.5 for definition of acronyms.

3.6. Validation studies

To satisfy cGMP protocols [26,27], certain analytical criteria must be tested, as below. The ABI CE instrument was used for these studies.

3.6.1. Linearity

Samples of eight MK-0677 solutions containing 0.32 μg/ml to 0.54 mg/ml (0.08% to 135% of the target concentration, 0.4 mg/ml) were injected in duplicate. The UV detector response at 200 nm was linear ($r^2 = 0.9997$) over the entire concentration range.

3.6.2. Limits of detection (LOD) and quantitation (LOQ)

The LOD is the concentration of sample injected producing a signal-to-noise ratio of 3. The LOQ is defined as (3×LOD). The LOD was found to be 0.32 μg/ml, so the LOQ was 0.96 μg/ml.

3.6.3. Accuracy

A 0.4 mg/ml solution of MK-0677 (which contained <0.1% of the *S*-isomer) was spiked with 0.35% (w/w) of its *S*-isomer (which contained <0.1% of the *R*-isomer). The spiked solution was injected 5 times. The average recovery of the *R*-isomer was 103%. HPLC of the same sample using

the chiral OVM column confirmed the accuracy of the CZE results.

3.6.4. Precision

The method precision was evaluated by making five consecutive injections of a 0.4 mg/ml solution of MK-0677 containing 0.35% of the *S*-isomer. The R.S.D. was 0.03% for the *R*-isomer and 8.9% for the *S*-isomer.

3.6.5. Ruggedness

The solution stability was evaluating by repeatedly assaying the same 0.4 mg/ml MK-0677 solution over a one month period. The assay R.S.D. ($n=6$) was 0.8% for the MK-0677. The capillary-to-capillary and electrolyte-to-electrolyte variations were evaluated using two different capillaries and three different preparations of BGE. The assay R.S.D. was 0.8% ($n=6$). Based on the day-to-day results, the R_s of the two enantiomers was >1.5, and the peak tailing factor (defined in Ref. [18] as the peak width at 5% of the peak height divided by twice the peak front half width at 5% of the peak height) was <1.5.

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