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Enantiomeric separation of an aryloxyphenoxypropanoic acid by CE and LC☆

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Abstract

A capillary electrophoresis (CE) and an high performance liquid chromatography (HPLC) chiral separation have been developed for an aryloxyphenoxypropanoic acid, 2-{4-[(7-chloro-2-quinoxalinyl)oxy]phenoxy}propanoic acid, a new antitumor agent. The racemic mixture is analyzed, without derivatization, as the free acids. The CE assay is based on inclusion complexation with hydroxypropyl-β-cyclodextrin. HPLC separation is achieved with a CSP column with the glycopeptide, teicoplanin, as the chiral selector. Both methods give baseline resolution to the *R*-and *S*-isomers. The methods were validated for assay and for optical purity assessment of the *R*-isomer. For assay, the HPLC method is precise (RSD < 0.6%), accurate (error, 0.5%) and linear ($r^2 = 0.9998$). It is able to precisely (RSD = 0.5%) and accurately (error, 0.9%) detect 0.3–6.0% of one isomer (*S*) in the other (*R*). The CE assay is much less precise and accurate than HPLC. It is a good alternative to separate and detect the enantiomers, however.

Keywords: Aryloxyphenoxypropanoic acid; Chloroquinoxalinyloxyphenoxypropanoic acid; Chiral separation; Capillary electrophoresis; High-performance liquid chromatography; Hydroxypropyl cyclodextrin; Teicoplanin

1. Introduction

2-Aryloxyphenoxypropanoic acids (APPAs) have been widely used as herbicides [1–5]. 2-{4-[7-Chloro-2-quinoxalinyl)oxy]phenoxy}propanoic acid (XK469, A, Fig. 1), the 7-chloro analog of the herbicide Quizalofop, has been patented as an anticancer agent [6]. Though recent data suggest that the *S*-isomer is biologically more active, it is also more toxic than the *R*-isomer [7]. For this reason, the *R*-isomer is currently being developed as a potential antitumor agent by the National Cancer Institute. In order to assess the chiral purity, chiral stability, and pharmacokinetics of the isomers, an enantiomeric separation is needed. Our aim is to develop a simple and direct RP-

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HPLC or capillary electrophoresis (CE) enantiomeric separation for XK-469.

Chiral separation of native 2-phenoxy propanoic acids (PPAs) has been achieved with CE using cyclodextrin (CD) or its derivatives in the buffer electrolyte as the chiral selector [8–12]. Three points of interaction are required for enantioselectivity [13–15]. For CD to be a successful chiral selector, at least one substitutent of the analyte proximal to the asymmetric atom must be tightly interacting with the CD cavity while two other points of interaction can be established between the CD rims and two other substituents of the guest molecule [16–18]. In the separation of PPA, Gadre and Connors [19] showed that the carboxylic acid group is too polar to be the favored binding site for inclusion in the CD cavity. They suggested the phenoxy group is the substituent that preferrably binds to the CD cavity. The methyl and the carboxylic acid groups, in close proximity, readily interact with the CD rim, which results in the chiral recognition of PPA enantiomers. XK469 (Fig. 1, A) has pK_a's of 3.6 and 0.3. It is only sparingly soluble in water but insoluble in acid and tends to form micelles or molecular aggregates in aqueous solution. For ionized APPA such as XK469, the carboxylate is too polar for the CD cavity and the large lipophilic quinoxalinyl ring, which is removed from the chiral center, would be the preferred group to enter and bind to the CD cavity. Therefore, chiral resolution of XK469 would be expected to be more difficult than for PPA. To achieve chiral resolution of APPA with CD, the PA end of the molecule must be facilitated to enter the CD cavity, allowing the phenoxy ring, which sits next to the chiral center to bind the cavity. This allows the methyl and carboxylic groups to be proximal to the CD rim to form the three point interation required for chiral recognition. Using appropriate CD, organic modifier, pH and counter ions of the buffer electrolyte, a baseline separation of the APPA by CE was finally achieved.

Because the developed CE separation requires 90 min and the reproducibility and accuracy of the assay are marginal (within day RSD = 5.6%, error, 2.8%), an inherently more reproducible and accurate HPLC assay was sought. Successful enantiomeric HPLC separations of native PPAs have been widely reported. Many used chiral stationary phase (CSP) such as Pirkle-type CSP

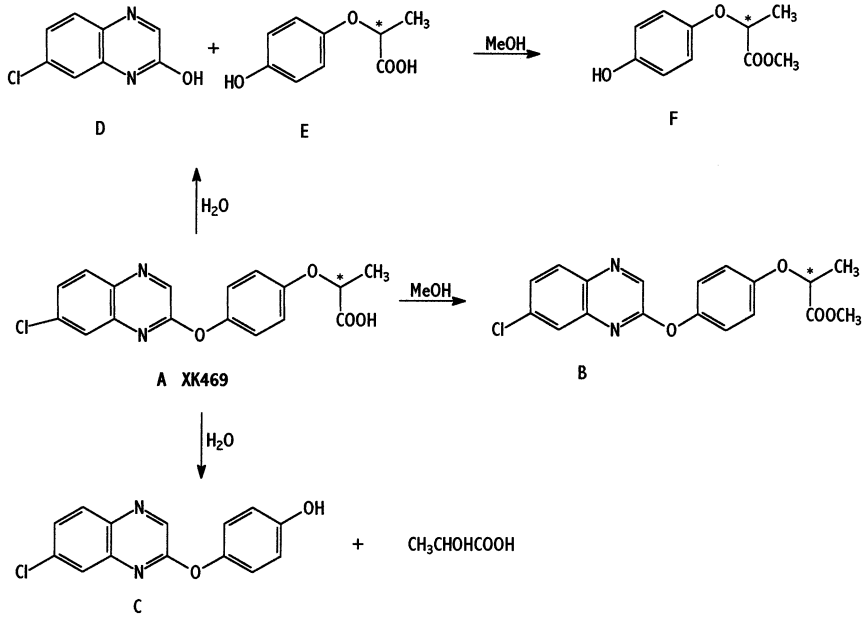

Fig. 1. Structure of XK-469 (A) and decomposition products.

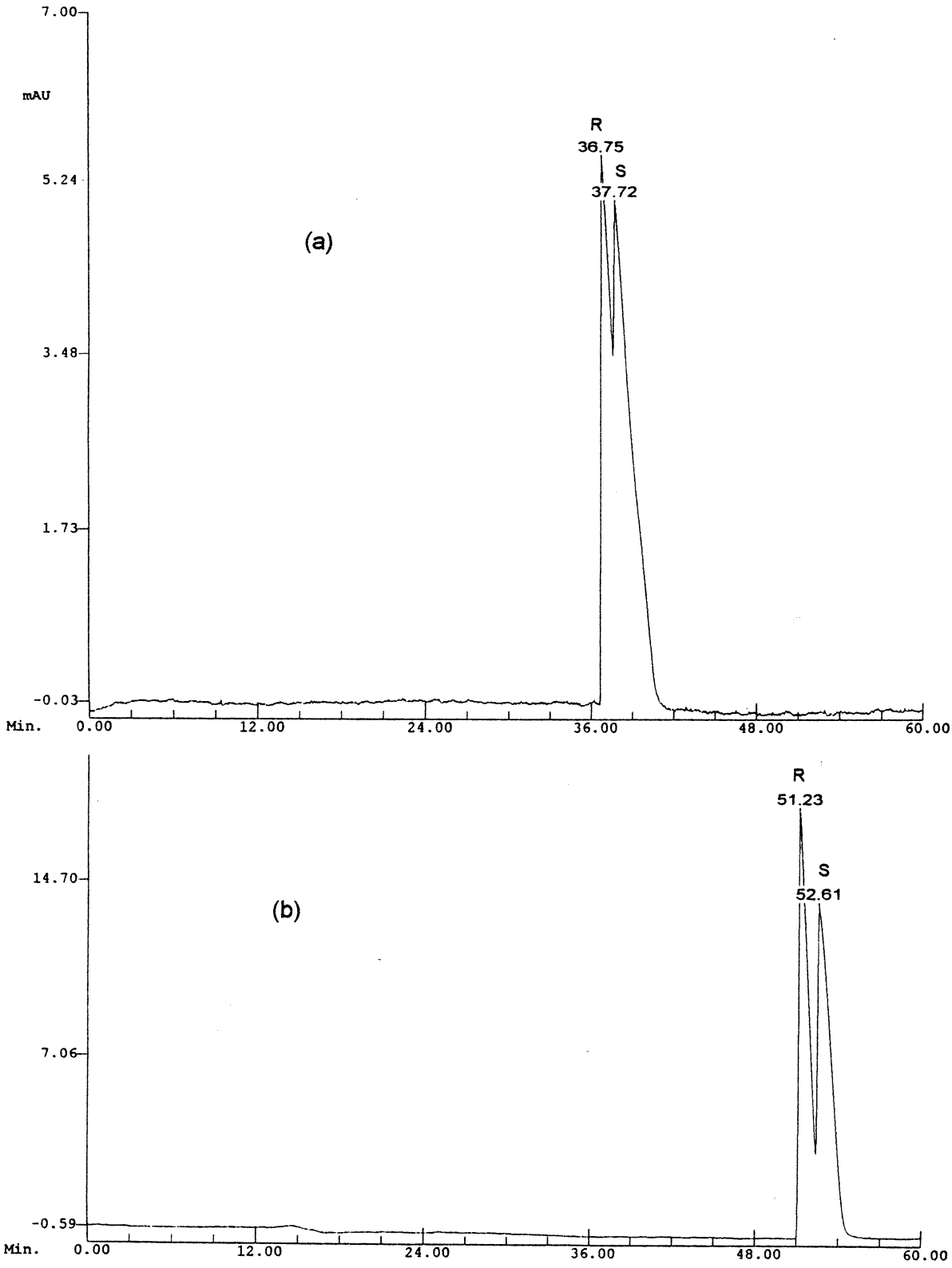

Fig. 2. Chiral separation of XK-469 using 20 mM HPCD in (a) 20 mM phosphate buffer at pH3; (b) 100 mM malonate buffer at pH 2.8. Capillary, coated, 45 cm (40.6 cm to detector), 50 μm i.d.; injection, 20 psi s; temperature, 20°C for cartridge, no control for carousel; voltage, 20 kV, − to +; detection, UV 240 nm.

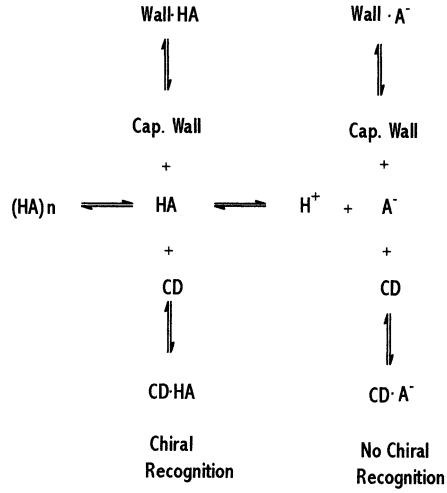

Fig. 3. Equilibrium scheme between XK469 (HA), its micelle (HA)*n*, the CD adduct (CD · HA) and the capillary wall adduct (wall · HA).

[20–22], α-acid glycoprotein [22,23], or macrocyclic glycoprotein CSP [26]. Others used ODS column with a chiral mobile phase [24,25]. Literatures on the enantiomeric separation of native APPAs are rare. Only the resolution of Fluazifop (2-{[5-(trifluoromethyl)-2-pyridinyl]oxy}-phenoxy) propionic acid), which employed an ODS column with a chiral mobile phase [27] and the separation of Quizalofop (the 6-chloro analog of XK469) which used a terguride CSP column [28], have been reported. Unfortunately, the terguride column is not commercially available. After numerous trials with many chiral columns, baseline resolution of the XK469 enatiomers was eventually achieved with a Chirobiotic CSP column.

This paper reports the development and validation of a CD–CE and a CSP–HPLC enantiomeric separation and assay of an APPA, XK469.

2. Experimental

2.1. Reagent

R-and *S*-XK469 isomers and the racemic mixture were received from the National Cancer Institute (MD, USA). The samples have been analyzed to be of high purity (> 99%). Analytical reagent grade NH_4NO_3, and HPLC grade MeOH were purchased from Mallinckrodt (Paris, KY, USA). Malonic acid and hydroxypropyl-β-cyclodextrin (HPCD, average molar substitution, 1.0) were purchased from Aldrich (Milwaukee, WI, USA). Water was purified through a Millipore Super-Q Pure Water System (Waltham, MA, USA).

2.2. Sample preparation

Test solution of the racemic mixture was prepared in MeOH (0.5 mg/ml). For assay validation of the *R*-isomer, five portions of the *R*-isomer (ranging from 0.5 to 3.0 mg) were individually weighed and dissolved in 5 ml aliquots of MeOH, yielding test solutions of 0.1–0.6 mg/ml of the *R*-isomer in MeOH. For optical purity assessment of the *R*-isomer or optical impurity (*S*-isomer) determination, stocks solutions of the *R*-isomer (1 mg/ml) and the *S*-isomer (60 μg/ml) were individually prepared in MeOH. The *S* stock solution was serially diluted to 30, 15, and 1.5 μg/ml with MeOH. Aliquots of the *R* stock were individually mixed with an equal aliquot of each of the five *S* solutions to yield test solutions containing 0.15–6.0% of the *S*-isomer in the *R*-isomer. Concentration of the *R*-isomer in these solutions was 0.5 mg/ml. For specificity demonstration, stock solutions of the racemic, and the *R*- and *S*-isomers were individually prepared in MeOH (1 mg/ml). The solutions were each diluted 1:1 individually with MeOH, 0.1 N HCl or 0.1 N NaOH. These test solutions were heated at 50–70°C for 1 h in a H2025 Temp-Blok Module Heater (Scientific Products, Division of American Hospital Supply Corp., McGaw Park, IL) to generate hydrolysis products. For possible thermal decomposition products, a solid sample of the racemic was also heated at 70°C for 24 h then dissolved in MeOH to yield a 0.5 mg/ml solution.

2.3. Capillary electrophoresis (CE)

CE was performed with a BioFocus 3000 CE System (Bio-Rad Laboratories, Hercules, CA, USA) using a coated capillary (Micro Solv CE-

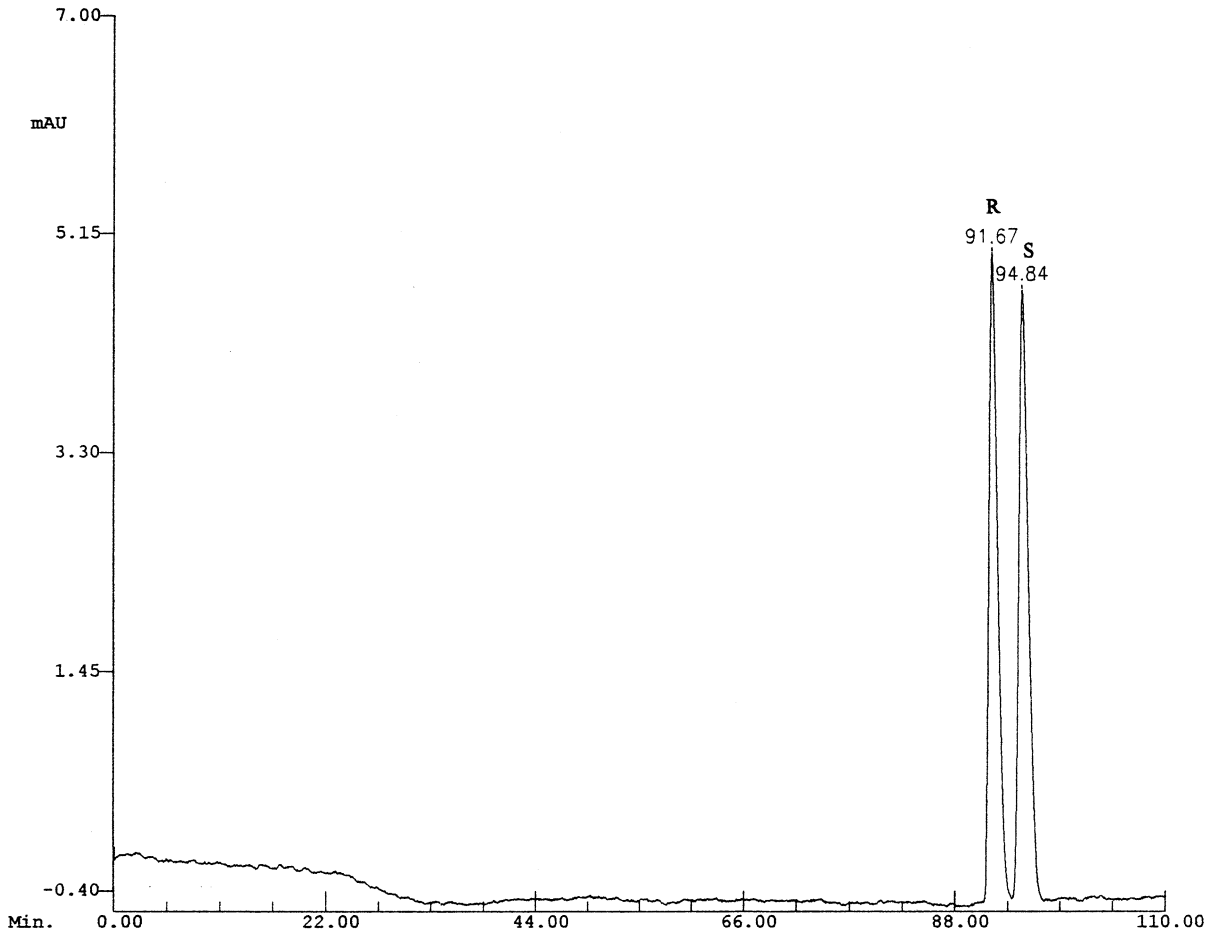

Fig. 4. Chiral separation of XK-469 using 20 mM HPCD in 20 mM malonate buffer at pH 2.8 with 30% methanol. Capillary, coated, 45 cm (40.6 cm to detector), 50 μm i.d.; injection, 20 psi s; temperature, 20°C for cartridge, no control for carousel; voltage, 20 kV, − to +; detection, UV 240 nm.

100SA Zero EOF) of 45 cm × 50 μm i.d. (40.6 cm to detector). Run buffer was 100 mM malonic acid, pH adjusted to 2.8 with NaOH, containing 20 mM HPCD and 30% (v/v) MeOH. Prior to each run, the capillary was washed, in sequence, with 30 s water, 60 s MeOH, 60 s water, and 90 s run buffer. Electrophoresis was performed at 20°C, with an applied voltage of 20 kV (− to +). Detection was towards the anode by UV at 240 nm. Injection was by pressure, 20 psi s. Test solutions were diluted 1–20 with water before analysis.

2.4. High-performance liquid chromatography (HPLC)

The HPLC system consists of a Varian (Walnut Creek, CA, USA) Vista 5500 HPLC pump, a Thermoseparation (Fremont, CA, USA) AS3000 Autosampler, and a Perkin Elmer (Norwalk, CT, USA) LC135 UV detector. Data were collected and analyzed with a HP (Mountain View, CA, USA) ChemStation for LC. Test solutions (10 μl) were directly injected onto an astec (Whippany, NJ, USA) Chirobiotic T, 250 × 4.6 ID stainless

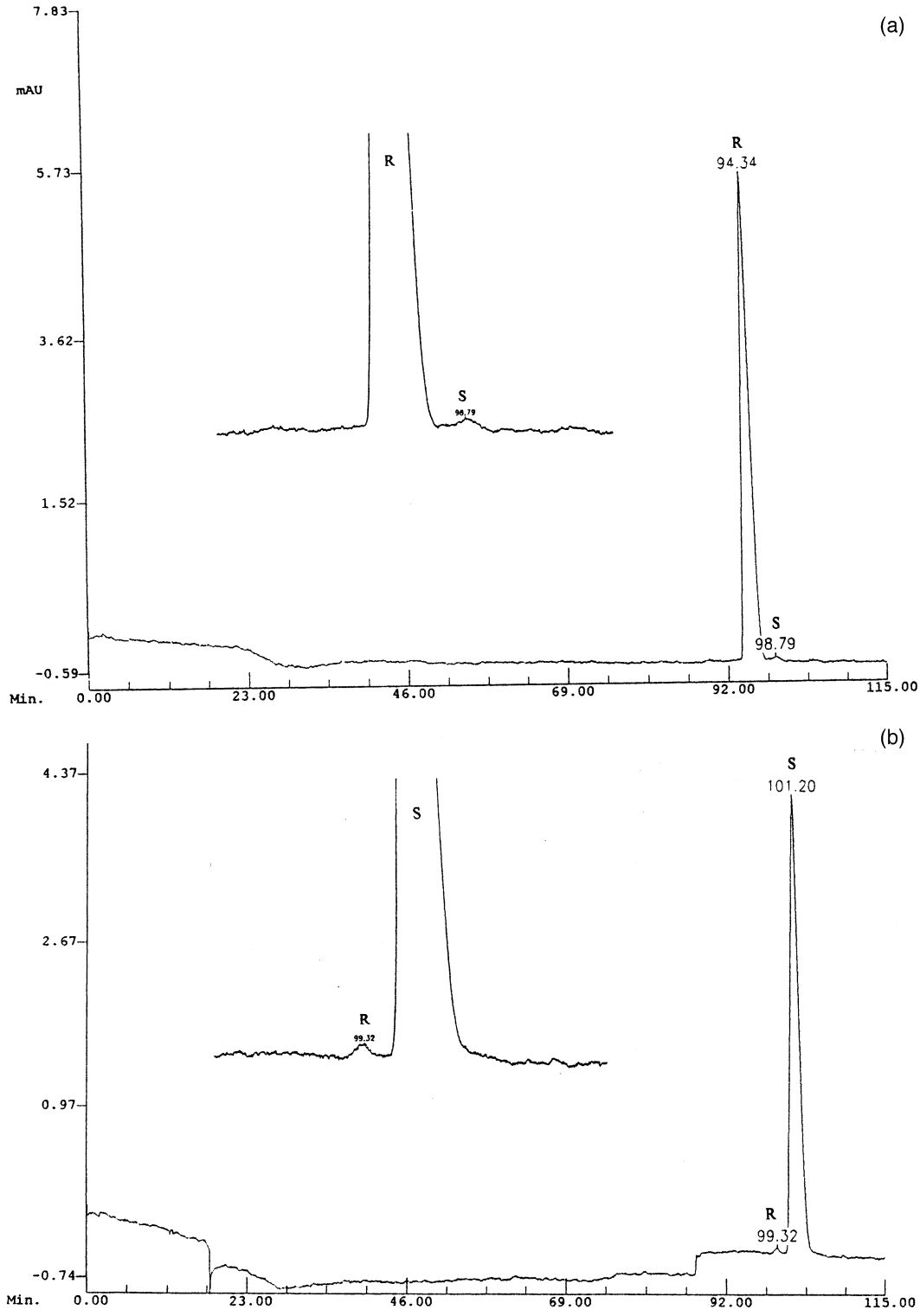

Fig. 5. Chiral separation of XK-469 with (a) 0.5% *R*-isomer in *S*-isomer and (b) 0.5% *S*-isomer in *R*-isomer. Experimental conditions are the same as in Fig. 4.

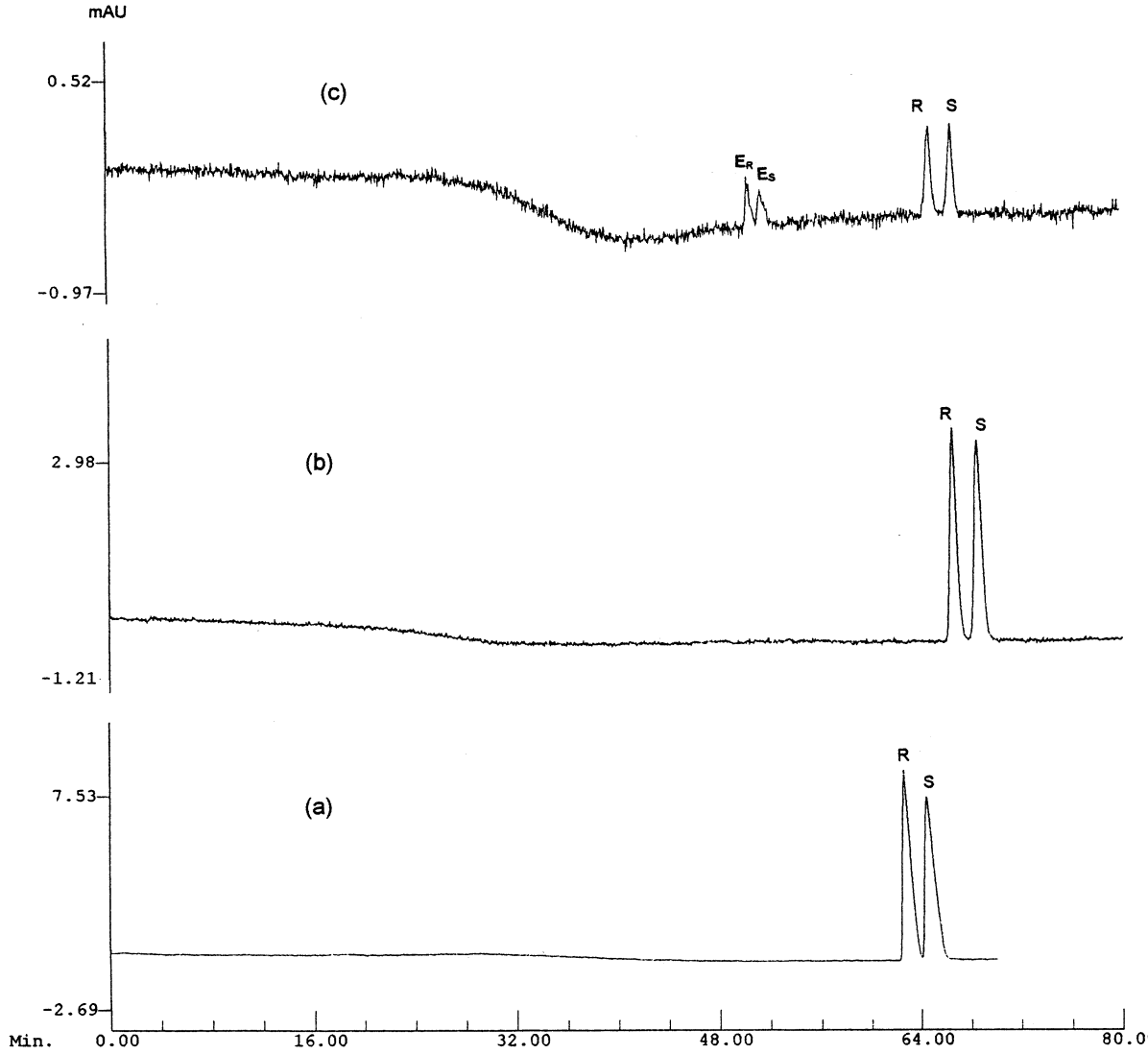

Fig. 6. Electropherogram of fresh (a) acid (b)/base (c) decomposed racemic XK469 (this figure was obtained with a different capillary, which resulted in migration time different from that of Figs. 4 and 5).

steel column. Elution was isocratic with a mobile phase of a mixture of water–MeOH (65:35, v/v) containing 20 mM NH_4NO_3. Detection was by UV at 255 nm.

3. Results and discussion

3.1. CE

CE using cationic CD derivatives as additive has recently been used effectively in resolving acidic enantiomers such as PPA and APPA [29]. The inavailability of these cationic CD derivatives at the time prompted our method development to concentrate on the commercially available α-, β-, γ-CD, dimethyl-β-CD (DMCD), trimethyl-β-CD (TMCD) and hydroxypropyl-β-CD (HPCD). With these neutral CDs, ionization of XK469 is needed for mobility. To allow the anodic detection of the analyte anion, a coated capillary was used to eliminate EOF. The pK_a of XK469 (3.6

Table 1
Validation results for assay of *R*-isomer (active drug)[a]

	CE	HPLC
Calibration range (%)	50–160%	50–160%
Calibration points	5	5
Linearity		
Slope (standard error)	168 023 (7591)	20 148 (159)
Intercept (standard error)	−1564 (2660)	94 (56)
Correlation coefficient (r^2)	0.9939	0.9998
Accuracy (% error)	2.8	0.5
Repeatability ($n = 6$, RSD %)	5.6	0.37
Intermediate precision ($n = 18$, RSD %)	14.3	0.59

[a] By external standard experiments.

Table 2
Optical purity assessment (determination of *S*- in *R*-isomer)[a]

	CE	HPLC
Calibration range (%)	0.3–6.0%	0.3–6.0%
Calibration points	4	4
Linearity		
Slope (RSD %)	0.9546 (0.03831)	0.9506 (0.002386)
Intercept	−0.0871 (0.1323)	0.0008 (0.0000824)
Correlation coefficient (r^2)	0.9968	1
LOD–LOQ		
Limit of detection	25 ng/ml	2 ng
Limit of quantitation	120 ng/ml	80 ng
Accuracy (% error)	15.0	0.9
Repeatability ($n = 6$, RSD %)		
0.15%	23.0	2.4
3.00%	3.4	0.5
Intermediate precision ($n = 18$, RSD %)		
0.15%	39.7	2.1
3.00%	28.4	0.9

[a] Calculated as area ratio of *S*/*R*.

[30]) and the stability concern of the capillary restrict the run buffer pH to above 2. With 15 mM cyclodextrins in 20 mM phosphate buffer at pH 3, 5, and 7, α-, β-, γ-CD, and DMCD gave no separation for the enantiomers. TMCD and HPCD did not yield separation either at pH 5–7. However, at pH 3, partial resolution of the enantiomers was observed with both TMCD and HPCD. This suggested that chiral recognition resulted from binding of the unionized APPA onto the CD cavity, consistent with earlier discussion [19] that the carboxylate ion is too polar to enter the CD cavity. Varying the CD concentration (10–50 mM) or the buffer concentration (20–100 mM) only affected the migration time but did not affect the resolution significantly. Fig. 2a is a typical CE separation of the XK469 enantiomers obtained with a pH 3, 20 mM phosphate run buffer containing 20 mM HPCD. The shape of the peaks suggested that the poor resolution were due to their broad width and excessive tailing. Reducing the buffer pH slightly to 2.8 to increase unionized XK469 only increased the migration time but not the resolution.

Excessive tailing peaks in CE are thought to be caused by overloading and electromigrational dispersion (EMD). Reducing the sample concentration and loading did not improve the peak asymmetry. EMD, therefore, must have an impact on the asymmetry and hence the resolution of the peaks for the enantiomers. The generally adopted rule of EMD is that if the ratio ($R\mu$) of the mobility of the buffer co-ion to that of the analyte is >1, a tailing peak would be obtained. Conversely, a fronting peak would result [31–33]. The farther $R\mu$ is from 1, the more asymmetrical the analyte peak would be. In a given run buffer, $R\mu$ is inversely proportional to the molecular weights (MW) of the analyte and buffer co-ion and directly proportional to the molar fractions of their charged ions. In the pH 3 phosphate buffer, the $R\mu$ is 21. Reducing the $R\mu$ would be a logical step to improve the resolution of the enantiomers. A buffer of an acid with MW and pK_a similar to that of the analyte would have $R\mu$ of 1. Ingelse and co-workers [34] showed that buffer co-ions may also compete with the analyte for the CD cavity. A large co-ion such as benzoate has a negative impact on the selectivity of CD's. Thus smaller organic acids were chosen for the run buffer. Fig. 2b shows the CE separation of the XK469 enantiomers in a pH 2.8 100 mM malonic acid buffer where the $R\mu$ is about 10.5. Asymmetry of the first peak (*R*-isomer) was reduced to

three from nine of the phosphate buffer (Fig. 2a). At the same time, the resolution ($R_{\%}$) was improved from 70 to 24% ($R_{\%}$ is defined as the percentage of the height from the baseline to the valley over the height of the smaller peak [35], since the half-height width for fused peaks cannot be measured for conventional calculation of R_s). The plate number of the CE system (N) also was increased from 5700 to 25 900. Further manipilation of the $R\mu$ by using pH 2.8 buffers of acetobutyrate, methoxyacetate and citrate ($R\mu = 0.2$, 3.8 and 4.0, respectively) did not improve further on CE efficiency and resolution of the enantiomers. As predicted, slightly fronting peaks were seen with the acetobutyrate buffer while slightly tailing peaks were observed with the methoxyacetate and citrate buffers.

Since peak asymmetry was greatly improved by using the malonate run buffer, effort was switched to reduce peak width to further enhance peak resolution. Ionic ratio of the run buffer (100 mM) to that of the sample solution (0.14 mM) was > 500, electrostacking could not be further improved to reduce peak width. Increasing the plate number (efficiency) of the CE system would be the obvious next step to optimize the enantiomeric

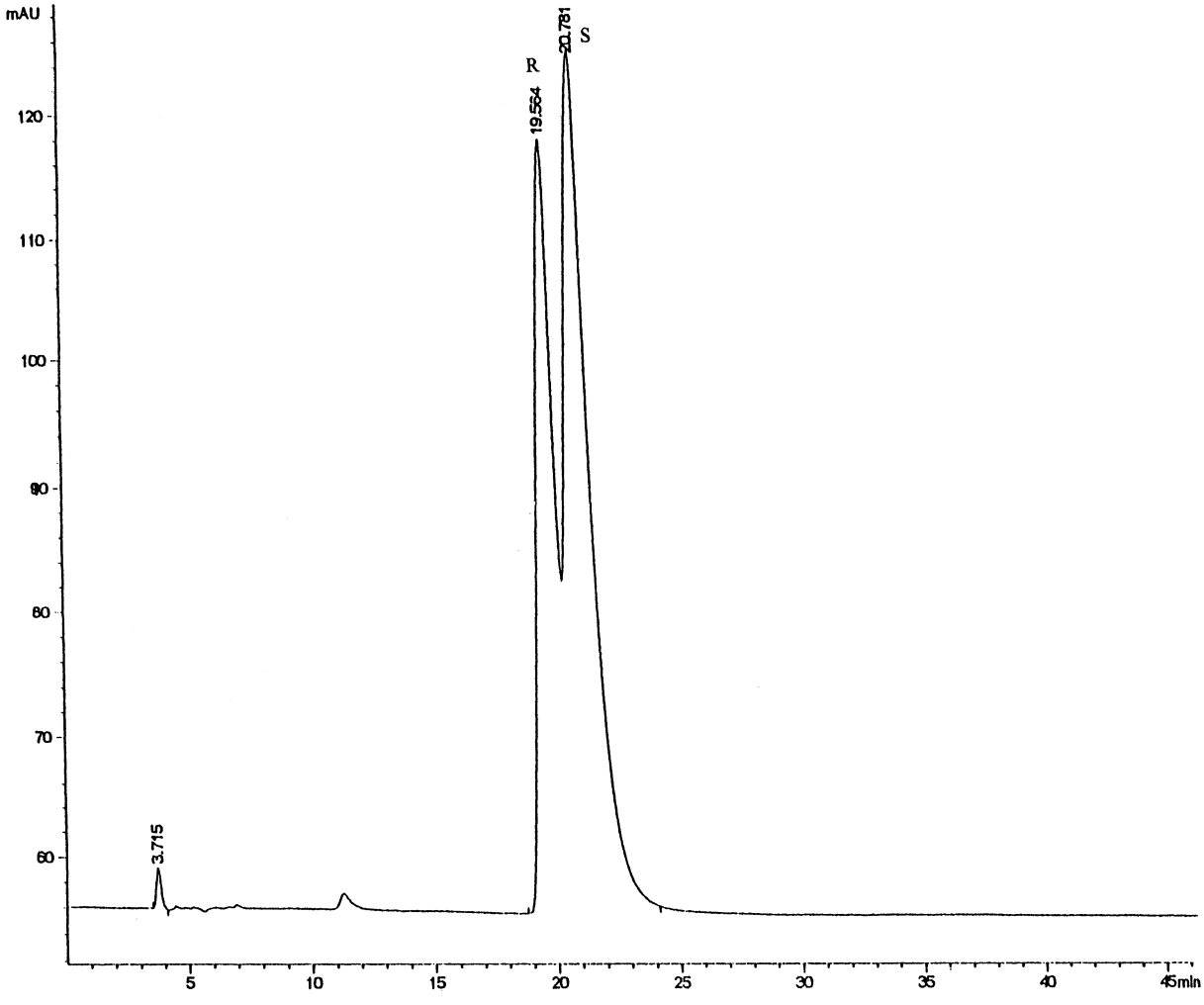

Fig. 7. Chiral separation for XK-469 in HPLC. Column, Chirex 3005, 250 × 4.6 ID; mobile phase, MeOH/H_2O (70/30) with 0.4% formic acid and 0.6% NH_4OH. Detection, UV at 255 nm.

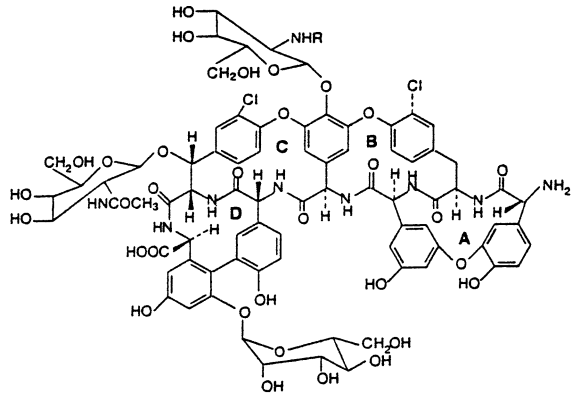

Fig. 8. Structure of teicoplanin, the stationary phase of a Chirobiotic T column.

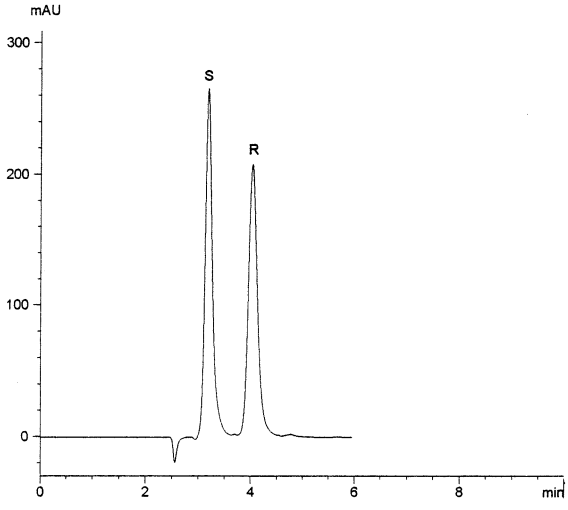

Fig. 9. Chiral separation for XK-469 in HPLC. Column, Chirobiotic T, 250 × 4.6 ID; mobile phase, mixture of water–MeOH (65:35, v/v) containing 20 mM NH_4NO_3. Detection, UV at 255 nm.

separation. During pK_a determination, we observed that XK469 formed molecular aggregates or micelles in neutral or acidic aqueous solutions. The lipophilic end of XK469 could also have affinity for the inside wall of the coated capillary. Fig. 3 depicts the equilibria between XK469 (HA), its micelle (HA)*n*, the CD adduct (CD · HA), and the capillary wall adduct (wall · HA) possibly affecting the CE process. Maximum chiral recognition would be achieved by optimizing the CD × HA population. Decreasing the buffer pH would only lengthen the already long migration time for the analytes (52 min, Fig. 2b). Instead, we added MeOH to the run buffer to shift the (HA)*n* and wall × HA to HA. Fig. 4 is the typical CE separation of XK469 using a pH 2.8 malonate buffer (100 mM) containing 20 mM HPCD and 30% MeOH. The peaks are baseline resolved with R_s of 1.8 ($R_s = 1.18\ \Delta t/\Sigma W_{1/2}$), peak asymmetry of 2 and plate number of 50 900. The plate number is somewhat lower than that expected of a typical CE system. This is probably due to diffusion of the analytes during the long (~ 90 min) migration process. Efforts to reduce the migration time by varying the run voltage, MeOH and salt concentration resulted in decreased resolution. Although, increasing the HPCD concentration to 30 mM and/or decreasing the buffer pH to 2.5 increased the R_s further, the migration times were increased to > 2 h. With consideration for run time, CE conditions chosen for the chiral assay and validation were those described for Fig. 4 and in Section 2.3. Fig. 5 shows the separation of each isomer containing 0.5% of the other. The method is specific and stability-indicating. Fig. 6 shows the separation of the analytes and their acid/base catalyzed decomposition products. XK469 has been shown [36] to decompose to UV detectable products B–F(Fig. 1) in methanolic–aqueous acid/base solutions.

Table 3
Effect of pH and methanol concentration on HPLC separation of *R*, *S*-XK469

(20 mM NH_4NO_3 in H_2O, pH 4)			
Methanol concentration[a]	*k′ S* isomer	α	Resolution (R_S)
25	3.48	1.52	2.10
30	2.76	1.57	2.22
35	2.16	1.59	2.54
40	1.56	1.69	2.69
(20 mM NH_4NO_3 in H_2O, 35% methanol[a])			
pH	*k′ S* isomer	α	Resolution (R_S)
4	2.16	1.59	2.54
5	2.12	1.64	2.45
6	1.76	1.83	2.74

[a] Methanol also contains 20 mM of NH_4NO_3.

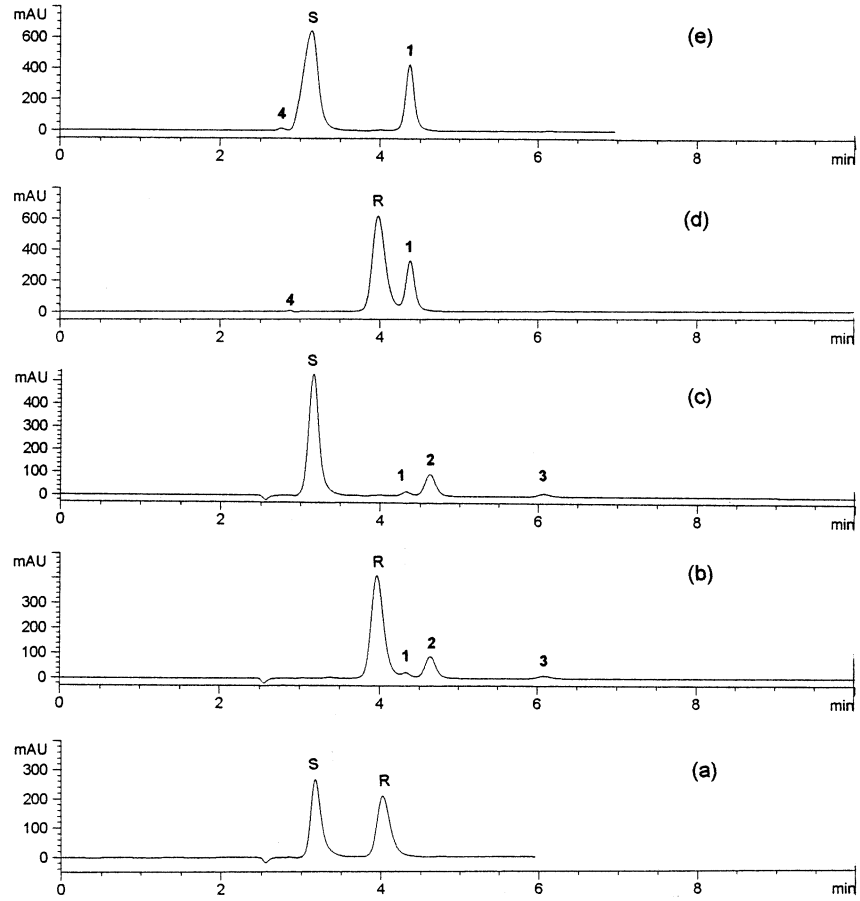

Fig. 10. Chromatogram of fresh and acid/base decomposed XK469. (a) Fresh solution of racemic XK 469; (b) *R*-isomer heated in acidic solution; (c) *S*-isomer heated in acidic solution; (d) *R*-isomer heated in basic solution; (e) *S*-isomer heated in basic solution.

Under the CE experimental conditions, only product E would be anionized and detected. Indeed, the only products observed by the CE from acid/base decomposed solutions of the *R*-, *S*-and racemic XK469 were the earlier migrating E in Fig. 6. The UV profiles of E_R and E_S are identical (single λ_{max} at 290 nm) and are consistent with that reported for E [36]. Electropherograms of decomposed solutions of the individual XK469 enantiomers confirmed that E_R and E_S are originated from *R*-XK469 and *S*-XK 469, respectively. Heating the bulk at 70°C for 24 h or heating the MeOH solution at 70°C for 2 h did not alter the electropherogram. Assay validation results are presented in Table 1 and Table 2. The assay for the *R*-isomer is reasonably precise (within day RSD < 6%), accurate (error, 2.8%) and linear ($r^2 = 0.9939$). The optical impurity determination at 0.3–6.0% of *S*-isomer in *R*-isomer has errors of 15% and within day RSD of 3.4 at 3% *S*- in *R*-isomer. Detection limits are 25 ng/ml for both isomers in the presence of 1000 times of their antipodes. Test solutions are stable for at least 6 days.

3.2. HPLC

Attempts to separate the XK469 enantiomers by HPLC using CSP columns based on cyclodextrins (Cyclobond I, II, III), Pirkle type (ChiraSphere and Chirex), protein type such as bovine serum albumin (BSA), and macrocyclic glyco-

protein (Chirobiotic V) were all met with marginal success. The best resolution ($R_{\%} = 30\%$) was achieved with a Phenomenex Chirex 3005 column (Fig. 7). It appears that the π–π interaction of the CSP (*R*-naphthylglycine and 3,5-dintrobenzoic acid amide) of the Chirex column with the quinoxalinoxy overshadowed that with the phenoxy ring of XK469, rendering insufficient chiral recognition. Since CSP columns are unstable at pH < 3 and the pK_a of XK469 is 3.6, for a successful resolution of the XK469 isomers, the CSP needs to have a base and an aromatic ring proximal to its chiral center. This allows the CSP to interact strongly with the chiral propanoate moiety of XK469. Availability of these features in the terguride based column is probably the reason for baseline separations of many APPA, including Quizalofop [28]. Unfortunately, the terguride based column is not commercially available. A careful examination of commercially available CSP columns suggests that the Chirobiotic T may provide enantiomeric separation of XK469. Teicoplanin (Fig. 8) is the CSP in the Chirobiotic T column. Its A region has a chiral center with an amine and a phenoxyphenyl substitution provides the chiral recognition environment for XK469. Using a mobile phase of 20 mM NH_4NO_3 (pH 4) containing 35% MeOH, a baseline separation of the XK469 enantiomers was achieved with the Chirobiotic T column (Fig. 9). The separation took only 6 min. Table 3 shows the effect of MeOH and pH on the retention and resolution of the enantiomers.

Using the conditions described in Section 2.4. The HPLC method was validated for assay of the *R*-isomer and for optical purity assessment (determination of *S* in *R*). The method is specific and stability-indicating. Fig. 10 shows the separation of the analytes and their acid/base catalyzed solvolysis products. Three products (**1**–**3**) are observed in the acid decomposed solution while two (**1** and **4**) were detected in the base decomposed solution. Product **4** can be significantly detected when the detection wavelength was switched to 290 nm. Spectral (UV) comparison of products **1**–**4** to those in a related manuscript [36] suggests that **3** and **4** have UV profiles distinct and different from that of XK469 and corresponds to D and E (Fig. 1), respectively. The UV profiles of **1** and **2** are similar to that of XK 469 and are consistent with B and C (Fig. 1). Heating the bulk at 70°C for 24 h and heating the MeOH solution at 70°C for 2 h did not generate detectable products. Assay validation results are presented in Tables 1 and 2. The assay for the *R*-isomer is precise (RSD < 0.4%), accurate (error, 0.5%) and linear ($r^2 = 0.9998$). The optical impurity determination at 0.3–6.0% of *S*- in *R*-isomer is accurate (0.9% error) and precise (within day RSD = 0.5%). Detection limits are 2 and 3 ng for both isomers, based on 3 X signal to noise ratio. Test solutions are stable for at least 6 days.

4. Conclusion

A CE and an HPLC enantiomeric separation of an APPA, 2-{4-[7-chloro-2-quinoxalinyl)oxy]-phenoxy}propanoic acid (XK469), the 7-chloro analog of the herbicide Quizalofop, have been developed. Both gave baseline resolution for the enantiomers. The methods have been validated for the assay of the *R*-isomer, the drug candidate, and determination of optical impurity (*S*-isomer). The HPLC method is superior to the CE method. It is faster (6 vs. 90 min), more precise (within day RSD 0.4 vs. 6.0%) and more accurate (error 0.5 vs. 2.8%). The CE method, however, is an alternative method to assess optical purity of the isomers without resorting to the expensive chiral HPLC column. If the precision and accuracy of CE quantitation were to be improved, an internal standard would be needed [37,38].

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