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Determination of enantiomers in a synthetic argininal peptide using capillary zone electrophoresis and high-performance liquid chromatography

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Abstract

SCH 201781 is a synthetic argininal peptide containing two chiral centers and an aromatic sulfonamide group. It can exist as four reversible forms, the aldehyde, the hydrate, and two diastereomeric aminals. Capillary zone electrophoresis (CZE) and reversed-phase high-performance liquid chromatographic (HPLC) methods were developed to separate and quantitate the enantiomers in SCH 201781. Comparable results were obtained using both methods. The CZE method uses direct injection, while the HPLC method requires a precolumn derivatization and is more time consuming. The CZE method provides superior sensitivity to the HPLC method. Both methods were shown to be precise and reproducible.

Keywords: Enantiomer separation; Diastereoisomer separation; Background electrolyte composition; Derivatization, LC; Peptides; Semicarbazide

1. Introduction

The past decade has seen a rapid proliferation in the use of CZE for the separation of chiral compounds [1,2]. The tremendous efficiencies obtained using CZE, as well as the wide array of chiral modifiers that can be added to the background electrolyte (BGE) make this technique extremely powerful for separating enantiomers. As indicated by recent reviews [3,4], inclusion of cyclodextrins (CDs) in the background electrolyte has been useful for many chiral separations. The introduction of anionic cyclodextrin derivatives by Terabe et al. in 1989 [5] allowed an opening of the ''retention window'' available in CD-electrokinetic chromatography (CD-EKC). The counter-current mobility of anionic CDs such as sulfated-β-CD [6,7], sulfobutylether-β-CD (SBE-β-CD) [8,9], sulfoethylether-β-CD [10,11], carboxymethyl-β-CD [12,13] counteracts the electroosmotic flow (EOF) which drives analyte towards the cathode and, typically, the detector. When an analyte forms a complex with anionic chiral selectors, it moves away from the detector, increasing its residency time in the capillary

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and the number of chiral selective interactions the analyte will have with the cyclodextrin derivative. Chankvetadze et al. [14], summarized the useful ramifications of this counter current phenomenon, particularly the ability to reverse the elution order of enantiomers.

The work presented here demonstrates two unique solutions (CZE and HPLC) for determining the enantiomeric composition of SCH 201781 (Fig. 1), also known as CVS 2044, a compound jointly studied by Schering-Plough and Corvas International [15]. It is an argininal peptide anticoagulant containing two chiral centers. It can exist as four reversible forms, the aldehyde, the hydrate, and two diastereomeric aminals [16]. Analysis of its enantiomeric content required either the separation of the individual forms and their respective enantiomers or derivatization to a single form with subsequent separation of enantiomer. Attempts to obtain enantiomeric separations of SCH 201781 by chiral HPLC were unsuccessful due to the number of forms simultaneously present. However, an achiral HPLC method to quantitate the enantiomers was developed by derivatizing the aldehyde form with a chiral semicarbazide to form diastereomers. This method requires tedious sample preparation, which is not conducive to the routine analysis required for batch release in pharmaceutical operations. A direct CZE method requiring no derivatization was also developed for measurement of enantiomers, allowing for more accurate quantitation due to superior separation between the enantiomers.

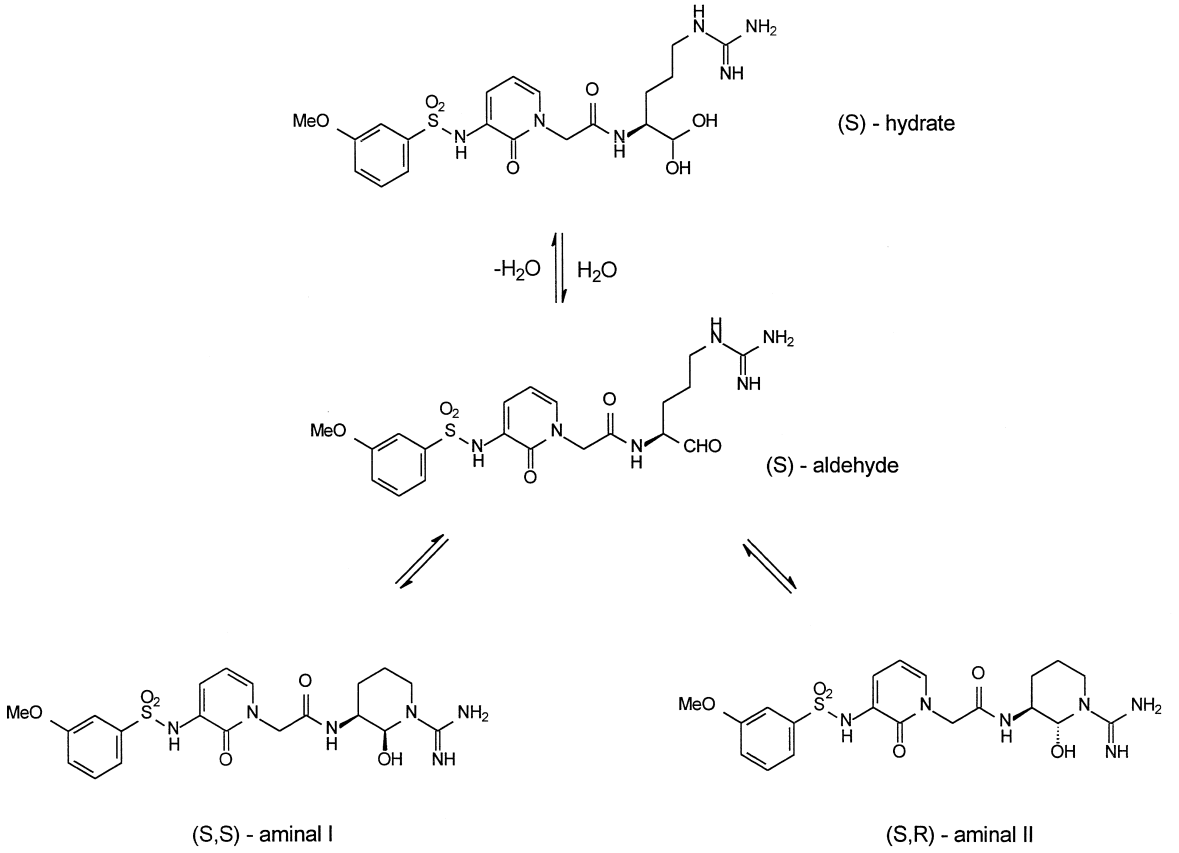

Fig. 1. Structure of equilibrium of four forms of SCH 201781.

2. Experimental

2.1. Reagents and chemicals

Citric acid, anhydrous, and trisodium citrate, dihydrate, were obtained from Fluka (Milwaukee, WI, USA). Dibasic potassium phosphate, sodium acetate, anhydrous, and methanol were obtained from Fisher Scientific (Springfield, NJ, USA). Sulfobutylether-β-cyclodextrin (SBE-β-CD), 4 sodium salt was obtained from Cydex (Overland Park, KS, USA). α-Cyclodextrin (α-CD), β-cyclodextrin (β-CD), γ-cyclodextrin (γ-CD), 2,6-di-*O*-methyl-β-cyclodextrin (di-OMe-β-CD), 2,4,6-tri-*O*-methyl-β-cyclodextrin (tri-OMe-β-CD), 2-hydroxy-propylated-β-cyclodextrin (OH-β-CD), buffers pH 2.5 (50 m*M* phosphate), pH 7.0 (50 m*M* phosphate), pH 8.3 (89 m*M* Tris, 89 m*M* boric acid), pH 9.3 (50 m*M* borate), phosphoric acid and CE-grade water were obtained from Hewlett-Packard (San Fernando, CA, USA). Ethanol (dehydrate 200 proof) was obtained from Pharmco (Brookfield, CT, USA). Methyl-β-cyclodextrin (Me-β-CD) was obtained from Sigma (St. Louis, MO, USA). Sulfated-β-cyclodextrin (sulfated β-CD) and 2-hydroxyethylcellulose were obtained from Aldrich (Milwaukee, WI, USA). (*S*)-and (*R*)-SCH 201781, and (*S*)-and (*R*)-benzylmethylsemicarbazide were prepared by Chemical Development in the Schering-Plough Research Institute.

2.2. Capillary electrophoresis

Capillary electrophoresis was performed on an Agilent^{3D} capillary electrophoresis system (Agilent Technologies, Palo Alto, CA, USA) equipped with a photodiode array detector and a fused-silica capillary (72 cm effective length ×75 μm I.D.) or a polyvinyl alcohol (PVA) coated capillary (56 cm effective length ×50 μm I.D.). All of the capillaries were obtained from Agilent Technologies. The detector window was located 7 cm from the cathodic end of the capillary. The capillary was rinsed for 2 min each with 0.1 *M* H_3PO_4, water and 50 m*M* pH 2.5 citrate buffer pH 2.5, and then rinsed for 5 min with the electrolyte prior to sample injection. The samples were injected hydrodynamically at 50 mbar for 5 s. The separation potential was maintained at −15 kV or −20 kV. The detection wavelength was 200 nm and the capillary temperature was 15°C.

The 50 m*M* citrate buffer was prepared by adjusting pH of a 50 m*M* citric acid solution to 2.50±0.02 using a 50 m*M* sodium citrate solution. The electrolyte was prepared by dissolving 20 m*M* SBE-β-CD and 5 m*M* β-CD or 6.5 m*M* di-OMe-β-CD in 50 m*M* citrate buffer solution. Samples were prepared in CE-grade water at concentrations of 1 or 2 mg/ml.

The percent of enantiomer content in SCH 201781 using the CZE method was calculated as follows:

$$\frac{\mathrm{CPA}_{(R,R)\text{-aminal I}}}{\mathrm{CPA}_{(R,R)\text{-aminal I}} + \mathrm{CPA}_{(S,S)\text{-aminal I}}} \cdot 100$$

The CPA (corrected peak area) was calculated as peak area/migration time.

2.3. HPLC

A Hewlett-Packard 1050 HPLC system equipped with an UV–Vis detector at 310 nm was used. The SCH 201781 derivatives were separated and quantitated using a MetaChem Hypersil BDS column (5 μm, 25 cm×4.6 mm I.D.), a mobile phase consisting of methanol –0.01 *M* phosphate buffer, pH 7.5 (21:29, v/v) at a flow rate of 1.0 ml/min. Column temperature was maintained at 30°C using a Jones column heater/chiller. All mobile phase solutions were filtered and degassed. The injection volume was 10 μl.

The sample was derivatized by reacting 20 mg of SCH 201781 and 13 mg of (*S*) or (*R*)-benzylmethylsemicarbazide in 0.4 ml of ethanol and 0.6 ml of deionized water followed by adjustment to pH 6 by addition of 120 μl of 2.1 *M* sodium acetate solution. The solution was stirred for 3 h at room temperature and 200 μl of reaction solution was diluted to 10 ml with methanol. The final concentration of the SCH 201781 derivative was approximately 0.4 mg/ml.

The percent of enantiomer content in SCH 201781 using the HPLC method is calculated as follows:

$$\frac{\text{peak area of } (R,S)\text{-semicarbazone}}{\text{peak area of } (S,S)\text{-semicarbazone} + \text{peak area of } (R,S)\text{-semicarbazone}} \cdot 100$$

3. Results and discussion

3.1. CZE separation of enantiomers

The optimized separation of six components of SCH 201781, one pair of enantiomers for each of the aminals and the hydrate, is shown in Fig. 2. The aldehyde form readily converts to other forms in aqueous solution, and therefore is not observed under normal conditions. The effects of BGE buffer pH, chiral selector additives, the concentration of buffer and chiral selectors, and capillary temperature were studied. The parameters studied include the migration time (t_1) of the first peak of each enantiomeric pair, selectivity and resolution. Selectivity (α) is calculated using $\alpha = t_2/t_1$ where t is the migration time of each enantiomer. Resolution (R_s) is calculated using $R_s = 2(t_2 - t_1)/(w_2 + w_1)$; w represents the peak width at baseline.

3.1.1. Effect of background electrolyte buffer pH

The first step in method development was the evaluation of various BGE buffers at different pHs, i.e., 50 m*M* phosphate at pH 2.5 and 7.0, 50 m*M* citrate at pH 2.5, 89 m*M* borate–Tris at pH 8.3, and 50 m*M* borate at pH 9.3, using positive polarity in the CZE for analyte migration to the cathode. Adequate migration times were observed using 50 m*M* phosphate buffer at pH 2.50. At this pH, SCH 201781 has only one positive charge due to the

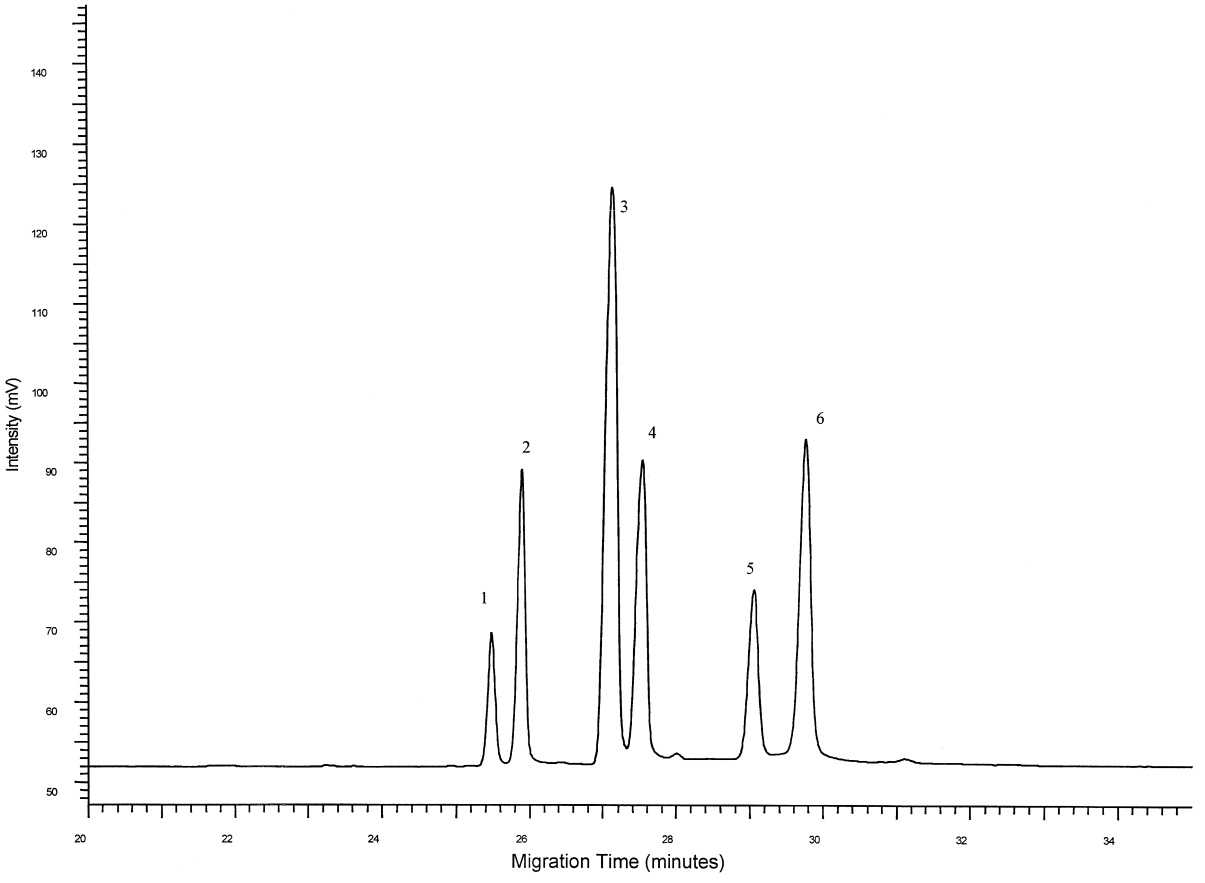

Fig. 2. CZE enantiomeric separations for three forms of SCH 201781. Background electrolyte: 50 m*M* citrate buffer at pH 2.50 with 20 m*M* SBE-β-CD and 5 m*M* β-CD; PVA coated capillary, 56 cm effective length ×50 μm I.D.; applied voltage, (−) 20 kV, 40 μA; injection, 250 mbar s (hydrodynamic mode) of 1 mg/ml SCH 201781 enantiomeric mixture; detection, 200 nm. Peaks: 1, (*R*,*R*)-aminal I; 2, (*S*,*S*)-aminal I; 3, (*S*,*R*)-aminal II; 4, (*R*,*S*) aminal II; 5, (*R*)-hydrate; 6, (*S*)-hydrate.

protonation of the sulfonamide group (pK_a of 7.4). As expected, the use of the BGE buffers without cyclodextrins resulted only in separation of the hydrate and two aminal forms, but no separation of enantiomers.

When SBE-β-CD was subsequently added as a chiral selector to the BGE buffer, the enantiomeric separations were similar using either phosphate or citrate buffer at pH 2.5. However, the current generated using phosphate buffer (>100 μA) is much higher than the current associated with citrate buffer (<65 μA). The higher current could cause irreproducible results and thus pH 2.5 citrate buffer was chosen as the BGE buffer.

3.1.2. Effect of chiral selector additives

Various chiral selector additives were added to the 50 m*M* citrate buffer at pH 2.5. Chiral separation was not achieved using only non-charged cyclodextrins, such as α-CD, β-CD, γ-CD, Me-β-CD, di-OMe-β-CD, tri-OMe-β-CD or OH-β-CD. However, the addition of neutral CDs resulted in a slight increase in migration times when operating in positive polarity mode operation. This is most likely due to the complexation of the SCH 201781 with the various CDs. The resulting complexes have greater hydrodynamic radii than the free forms of SCH 201781; therefore, the electrophoretic velocities of the complexes are lower. Since no separation of enantiomers was observed using neutral CDs alone, it can be assumed that the difference in the strength of inclusion for the enantiomers of each form of SCH 201781 is negligible.

The addition of sulfobutylether-β-CD (SBE-β-CD), a negatively charged derivative of β-CD with an average degree of substitution of four, to pH 2.5 buffer yielded an enantiomeric separation for each of the three forms of SCH 201781 (Fig. 2). The assignments for the aminal and hydrate peaks are based on UV–Vis spectra. However, the indicated order of elution for the aminal I and aminal II peaks is arbitrary and not confirmed. The assignment of elution order for the enantiomeric peaks (e.g., *R*,*R*- versus *S*,*S*-) was determined by spiking (*R*)-SCH 201781 with a sample of synthesized enantiomer, (*S*)-SCH 201781. The SBE-β-CD reversed the migration direction of analytes; therefore, detection was performed at the anodic end of the capillary.

It has been shown that sulfated cyclodextrins can separate enantiomers by a process of ''counter-current'' flow of the negatively charged additive with respect to the EOF, or, in this case, electrophoretic flow [17,18]. The opposing flow vectors result in an increase in the residency time of the analyte in the capillary, and thus, an increase in the number of chiral-selective interactions with the CD. It is likely that the separation is achieved due to the combination of the ''counter-current'' flow and the increased stability of the inclusion of the analytes with the anionic SBE-β-CD, compared to the neutral CD's screened. Certainly, the stabilizing effect of the charge such as the charge interaction between the various forms of SCH 201781 and SBE-β-CD will enhance the stability of the inclusion complex. No chiral separation was achieved using the more highly charged sulfated β-CD indicating that the spatial orientation of the negative charges around the β-CD cavity affects the strength of the inclusion complexes formed with various enantiomers. Other researchers have observed similar results [19].

The aminal II enantiomeric pair is not completely resolved using SBE-β-CD alone. The introduction of a second chiral selector enables baseline separation of all six peaks of interest (Fig. 3, curves b and d). Satisfactory separations were achieved using the combination of 20 m*M* SBE-β-CD with 5 m*M* β-CD or 6.5 m*M* di-OMe-β-CD. The elution order of the second aminal enantiomeric pair is reversed in the presence of di-OMe-β-CD, compared with SBE-β-CD alone or SBE-β-CD in combination with β-CD. The enantiomeric separation was not enhanced using 20 m*M* SBE-β-CD with either Me-β-CD or tri-OMe-β-CD at 2–20 m*M*, the concentration range studied. It has been reported that the combination of negatively charged CDs with uncharged CDs can provide adequate mobility with improved enantioselectivity [20,21].

3.1.3. Effect of buffer concentration

The effect of pH 2.5 citrate buffer concentration with 25 m*M* SBE-β-CD on the enantiomeric separations was studied. As shown in Table 1, the selectivity remains unchanged in the range of 10 m*M* to 100 m*M*, whereas the peak shape improves due to increased sample stacking. A 50 m*M* citrate buffer

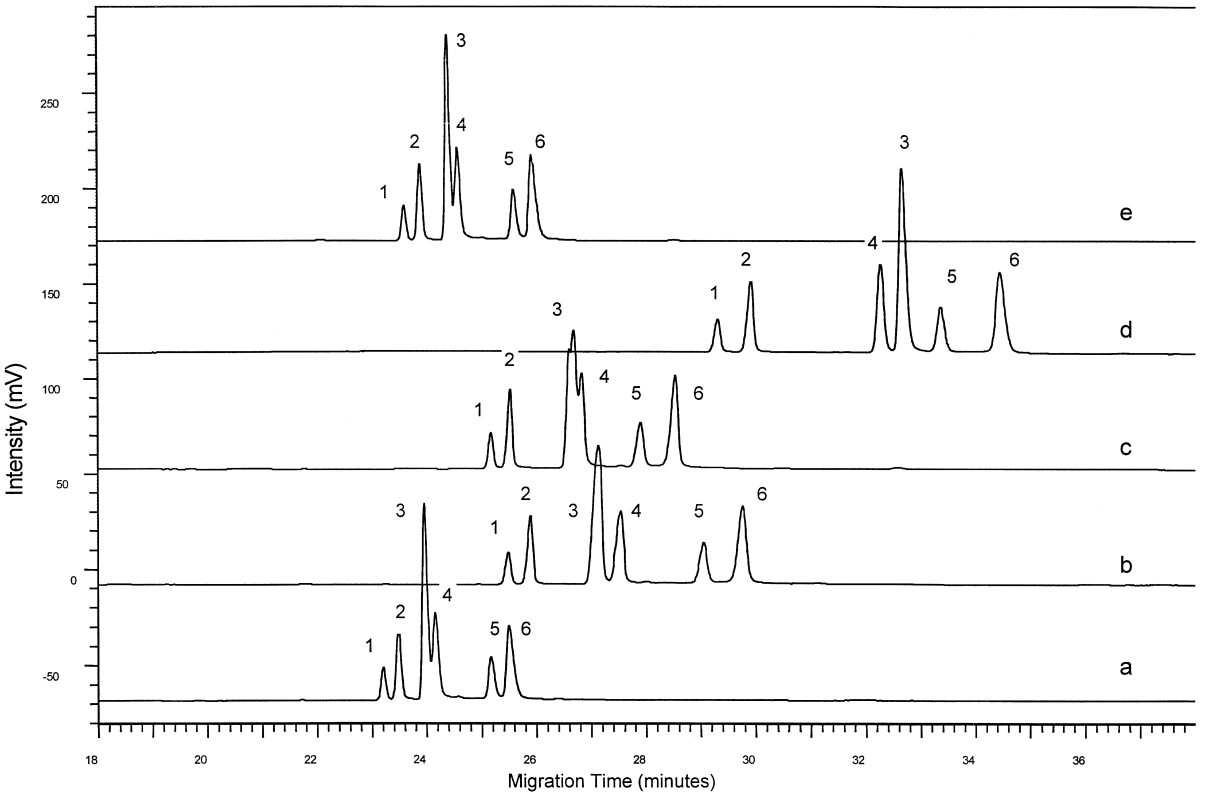

Fig. 3. Effects of chiral selector on CZE enantiomeric separations for three forms of SCH 201781. Background electrolyte: 50 m*M* citrate buffer at pH 2.50 containing (a) 20 m*M* SBE-β-CD; (b) 20 m*M* SBE-β-CD and 5 m*M* β-CD; (c) 20 m*M* SBE-β-CD and 5 m*M* Me-β-CD; (d) 20 m*M* SBE-β-CD and 6.5 m*M* di-OMe-β-CD; (e) 20 m*M* SBE-β-CD and 5 m*M* tri-OMe-β-CD, Peaks: 1, (*R*,*R*) aminal I; 2, (*S*,*S*)-aminal I; 2, (*S*,*S*)-aminal I; 3, (*S*,*R*)-aminal II; 4, (*R*,*S*)-aminal II; 5, (*R*)-hydrate; 6, (*S*)-hydrate. For other CZE conditions and sample concentration refer to Fig. 2.

was chosen for optimum peak shape while avoiding complications of high current or capillary clogging.

3.1.4. Effect of chiral selector concentration

The effect of chiral selector concentrations was evaluated, including SBE-β-CD alone and β-CD or di-OMe-β-CD in the presence of 20 m*M* SBE-β-CD (Table 2). Although the EOF is negligible for a fused-silica capillary at pH 2.5, a PVA coated capillary was used in these studies to completely eliminate EOF. Similar enantiomeric separations were achieved using both types of capillary.

As the SBE-β-CD concentration increases from 10 to 20 m*M*, the resolution of the aminal II enantiomers improves from 0.6 to 1.3, but then remains unchanged at higher concentrations. Resolutions for the aminal I and hydrate enantiomers do not vary significantly through the concentration range studied. Similarly, the selectivity between the three enantiomeric pairs is not affected significantly as SBE-β-CD concentration increases.

In contrast, an increase in the concentration of β-CD in the presence of 20 m*M* SBE-β-CD results in improved enantiomeric resolution, as shown in Table 2. The elution orders for the enantiomeric pairs remain unchanged throughout the concentration range studied.

The resolution of the aminal I enantiomers is also improved as the concentration of another neutral selector, di-OMe-β-CD, increases. The (*S*,*R*)-aminal II compound forms a stronger complex with di-OMe-β-CD compared to the other forms of SCH 201781, resulting in elution order changes throughout the concentration range studied (Fig. 4). As the di-OMe-

Table 1
The effect of concentration of background electrolyte citrate buffer[a]

Buffer concentration (m*M*)	Component[b]	Migration time (min)	Selectivity, α	Enantiomeric resolution, R_s
10	Aminal I	38.61	1.01	0.98
	Aminal II	39.31	1.01	0.97
	Hydrate	40.61	1.01	1.00
25	Aminal I	39.19	1.01	1.14
	Aminal II	39.96	1.01	0.96
	Hydrate	41.34	1.01	1.06
50	Aminal I	39.65	1.01	1.48
	Aminal II	40.57	1.01	0.96
	Hydrate	42.11	1.01	1.21
100	Aminal I	42.97	1.01	2.00
	Aminal II	44.26	1.01	0.77
	Hydrate	46.24	1.01	1.33

[a] CZE conditions: background electrolyte, pH 2.50 citrate buffer with 25 m*M* SBE-β-CD. fused-silica capillary, 72 cm effective length×75 μm I.D.; applied voltage, (−) 15 kV; injection, 250 mbar s (hydrodynamic mode) of 2 mg/ml SCH 201781 enantiomeric mixture; detection, 200 nm.
[b] See Fig. 1 for structures.

β-CD concentration increases to 10 m*M*, (*S*,*R*)-aminal II coelutes with (*R*)-hydrate, the enantiomer of the hydrate form.

Typically, when running in positive mode, analyte migration times increase as the SBE-β-CD concentration increases [14,22,23] and one would expect the opposite effect using reversed polarity mode. An increase in the concentration of SBE-β-CD should result in an increase in the concentration of the inclusion complex, which should decrease the migration time in the reversed polarity mode. Even if the various forms of SCH 201781 interact with SBE-β-CD by ion-pairing in addition to inclusion, the migration time should still decrease. However, as the concentration of SBE-β-CD increases, the migration time for all the peaks increases significantly. To further investigate whether this phenomenon is due to the increase of viscosity, various amounts of 2-hydroxyethyl cellulose were added to the BGE to mimic the viscosity increase of increased chiral selector concentration. The results show that as the concentration of 2-hydroxyethylcellulose increases, the migration time remains relatively unchanged, and thus it is concluded that a viscosity increase due to the higher chiral selector concentration is not the primary cause for longer migration times.

3.1.5. Effect of capillary temperature

It is well known that elevated or reduced capillary temperatures alter viscosity, electro-osmotic flow, and analysis time [24]. Temperature variation in the presence of SBE-β-CD can also change the chemical equilibrium and kinetics of the inclusion process, resulting in variations in the enantiomeric separation. The effects of capillary temperature on separation are shown in Table 3. As the temperature increases, the migration time and the resolution decrease, but the selectivity remains unchanged. At 25°C, the separation deteriorated, and an artifact peak was generated for the hydrate enantiomeric pair. The data indicate 10°C as the ideal capillary temperature, but because of occasional capillary cassette condensation at 10°C, 15°C was chosen for the procedure.

3.2. HPLC separation of enantiomers

3.2.1. Derivatization

The aldehyde form of SCH 201781 reacts with (*S*)- or (*R*)-benzylmethylsemicarbazide to form diastereomeric pairs which can be separated using achiral HPLC. The reaction results in equilibrium shift from the hydrate and the aminal forms toward the aldehyde form with subsequent formation of the semicarbazone. The reaction is shown in Fig. 5. When the four isoforms of (*R*)- and (*S*)-SCH 201781 are reacted with (*S*)-benzylmethylsemicarbazide, they are converted to (*R*,*S*)- and (*S*,*S*)-SCH 201781 semicarbazones, respectively. Similarly, the (*R*,*R*)-

Table 2
The effects of chiral selector concentration

Chiral selector	Concentration (m*M*)	Component[a]	Migration time (min)	Selectivity, α	Enantiomeric resolution, R_s
SBE-β-CD	10	Aminal I	19.23	1.02	1.82
		Aminal II	19.99	1.01	0.61
		Hydrate	21.02	1.02	1.67
	20	Aminal I	22.89	1.01	1.85
		Aminal II	23.62	1.01	1.30
		Hydrate	24.82	1.01	1.62
	30	Aminal I	26.99	1.01	1.68
		Aminal II	27.87	1.01	1.30
		Hydrate	29.34	1.01	1.52
	40	Aminal I	31.16	1.01	1.61
		Aminal II	32.28	1.01	1.33
		Hydrate	34.06	1.01	1.50
β-CD+ SBE-β-CD[b]	2	Aminal I	24.08	1.01	1.92
		Aminal II	25.16	1.01	1.38
		Hydrate	26.65	1.02	1.84
	5	Aminal I	25.57	1.02	2.22
		Aminal II	27.22	1.02	1.48
		Hydrate	29.16	1.02	2.70
	10	Aminal I	27.96	1.02	2.38
		Aminal II	30.61	1.02	2.06
		Hydrate	33.29	1.03	3.26
	20	Aminal I	33.69	1.03	3.05
		Aminal II	38.74	1.03	2.80
		Hydrate	43.65	1.05	4.10
di-OMe-β-CD + SBE-β-CD[b]	2	Aminal I	24.79	1.01	2.29
		Aminal II	26.16	1.00	0.46
		Hydrate	27.36	1.02	2.50
	5	Aminal I	27.70	1.02	2.63
		Aminal II	30.12	1.01	1.08
		Hydrate	31.20	1.03	3.63
	6.5	Aminal I	29.35	1.02	3.02
		Aminal II	32.31	1.01	1.67
		Hydrate	33.40	1.03	4.03
	10	Aminal I	33.90	1.02	3.47
		Aminal II	38.45	1.02	2.14
		Hydrate	39.30	[c]	[c]

[a] See Fig. 1 for structures, CZE conditions see Fig. 4.
[b] SBE-β-CD concentration was kept as 20 m*M*.
[c] (*S*,*R*)-aminal II and (*R*)-hydrate coelute.

and (*S*,*R*)-SCH 201781-semicarbazones are formed by reaction of SCH 201781 with (*R*)-benzylmethylsemicarbazide [16].

3.2.2. HPLC separation of derivatized semicarbazones

The achiral reversed-phase HPLC method with precolumn derivatization was optimized for the separation of the semicarbazones diastereomers. Representative chromatograms of (*S*,*R*)/(*R*,*S*)-semicarbazone and (*S*,*S*)/(*R*,*R*)-semicarbazone are shown in Fig. 6. The formation of the semicarbazone with (*S*)- benzylmethylsemicarbazide is more desirable for more sensitive and accurate quantitation since the chiral impurity peak elutes first. As with all derivatizations to form a diastereomeric pair for

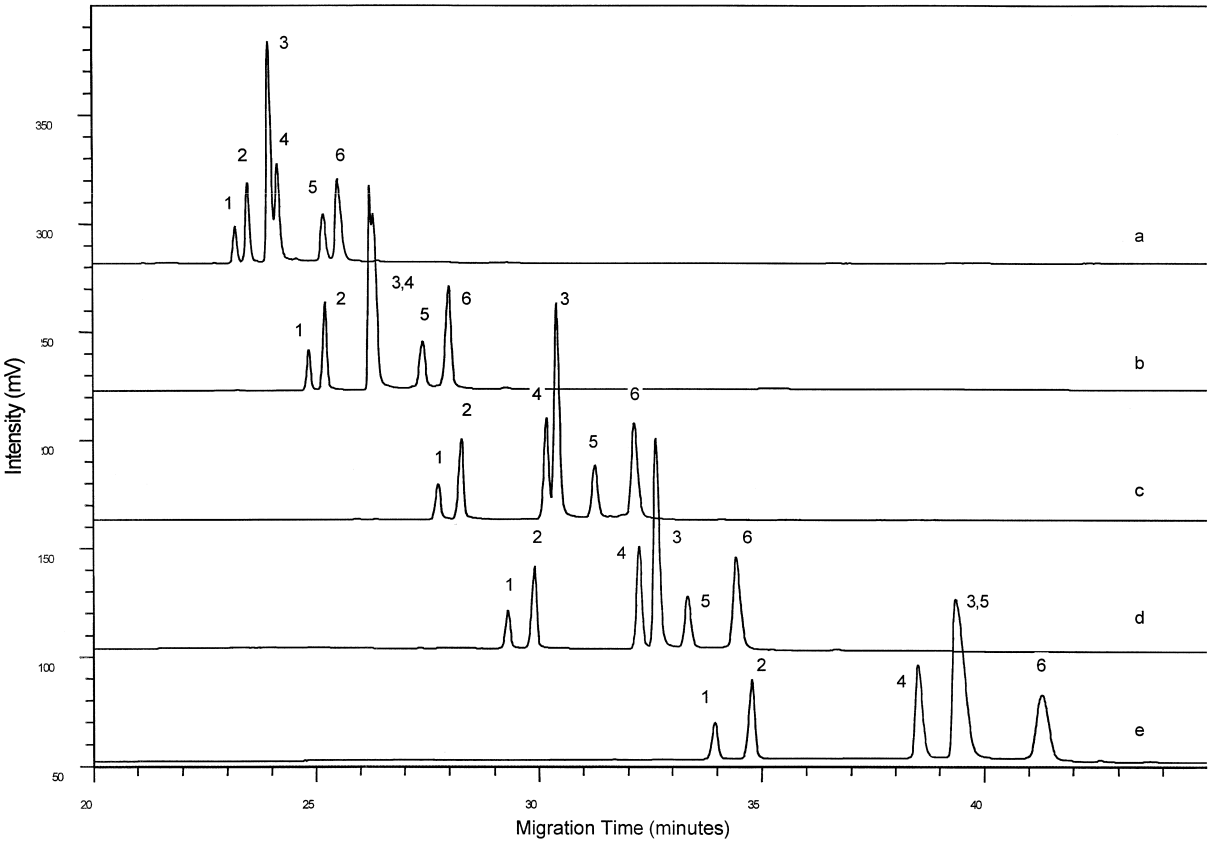

Fig. 4. Effect of di-OMe-β-CD concentration on CZE enantiomeric separations. Background electrolyte: 50 m*M* citrate buffer at pH 2.50 containing 20 m*M* SBE-β-CD and di-OMe-β-CD concentrations of (a) 0 m*M*; (b) 2 m*M*; (c) 5 m*M;* (d) 6.5 m*M*; (e) 10 m*M*. Peaks: 1, (*R*,*R*)-aminal I; 2, (*S*,*S*) aminal I; 3, (*S*,*R*)-aminal II; 4, (*R*,*S*)-aminal II; 5, (*R*)-hydrate; 6, (*S*)-hydrate. For other CZE conditions and sample concentration refer to Fig. 2.

enantiomeric determination, the chiral purity of the derivatization reagent is critical for accurate quantitation.

3.3. Comparison of CZE and HPLC method

3.3.1. Quantitative parameters

The system and method precision were established using both CZE and HPLC methods. System precision is demonstrated by five replicate injections, and method precision is illustrated by analyzing three sample preparations. As shown in Table 4, the CZE method is more precise than that of HPLC, and this is attributed to the better resolution and simpler sample preparation with the CZE.

CZE method accuracy is established by measuring the recovery of enantiomers at levels of 0.1%, 1% and 5%, in the presence of 100% SCH 201781 (Table 4).

Linearity was also established for enantiomer content of 0.1–8% using the CZE method, in the presence of 100% of SCH 201781 (Table 4).

The method sensitivities were determined by the limit of quantitation (LOQ, where the signal-to-noise ratio ≥ 10) and the limit of detection (LOD, where the signal-to-noise ratio ≥ 3). The superior separation obtained using the CZE method allowed use of a higher sample concentration (2 mg/ml) and resulted in a lower LOQ and LOD as compared to the HPLC (Table 4). The ratio of signal to noise was

Table 3
The effect of capillary temperature[a]

Capillary temperature (°C)	Component[b]	Migration time (min)	Selectivity, α	Enantiomeric resolution, R_s
11	Aminal I	42.87	1.01	1.48
	Aminal II	43.81	1.01	0.92
	Hydrate	45.42	1.01	1.22
15	Aminal I	39.65	1.01	1.48
	Aminal II	40.57	1.01	0.96
	Hydrate	42.11	1.01	1.21
20	Aminal I	34.80	1.01	1.28
	Aminal II	35.58	1.01	0.96
	Hydrate	36.89	1.01	1.04
25	Aminal I	32.79	1.01	0.96
	Aminal II	33.57	1.01	0.86
	Hydrate	34.80	1.01	0.71

[a] CZE conditions: background electrolyte, pH 2.50 citrate buffer with 25 m*M* SBE-β-CD. fused-silica capillary, 72 cm effective length ×75 μm I.D.; applied voltage, (−) 15 kV; injection, 250 mbar s (hydrodynamic mode) of 2 mg/ml SCH 201781 enantiomeric mixture; detection, 200 nm.
[b] See Fig. 1 for structures.

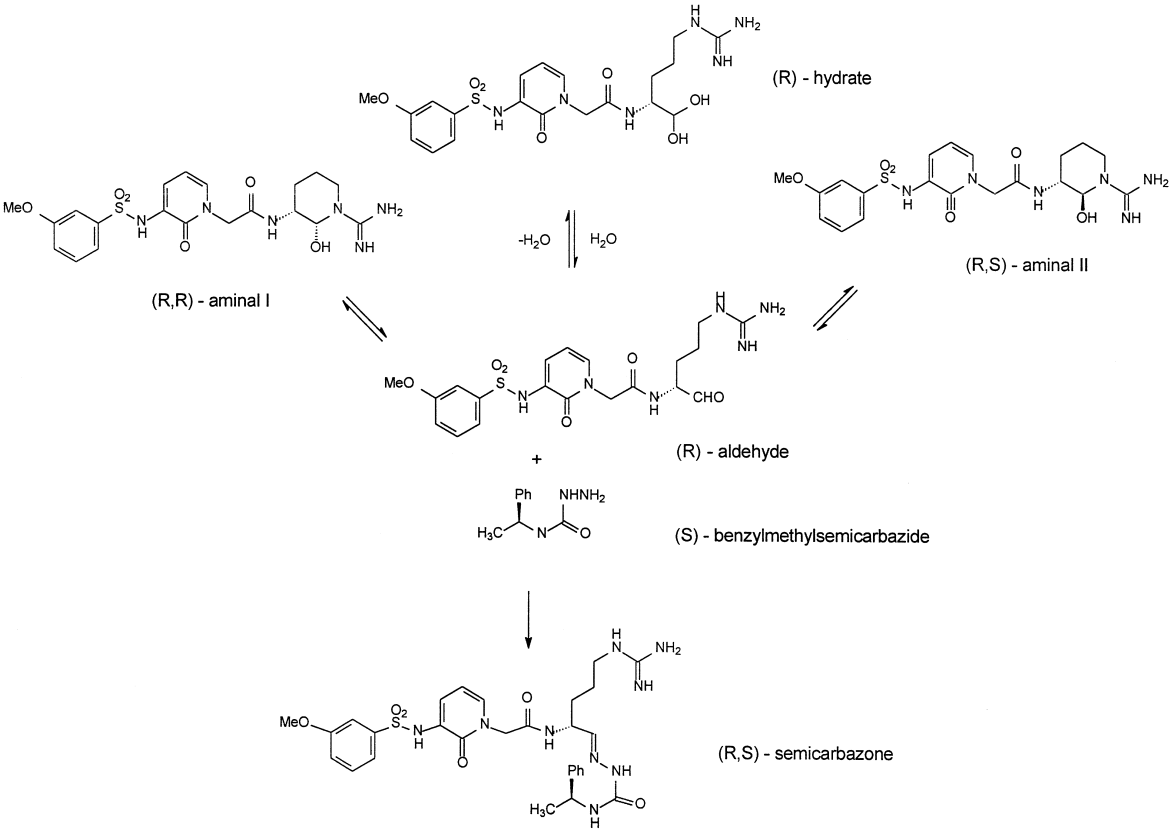

Fig. 5. Semicarbazone derivatization reaction scheme for SCH 201781 enantiomers.

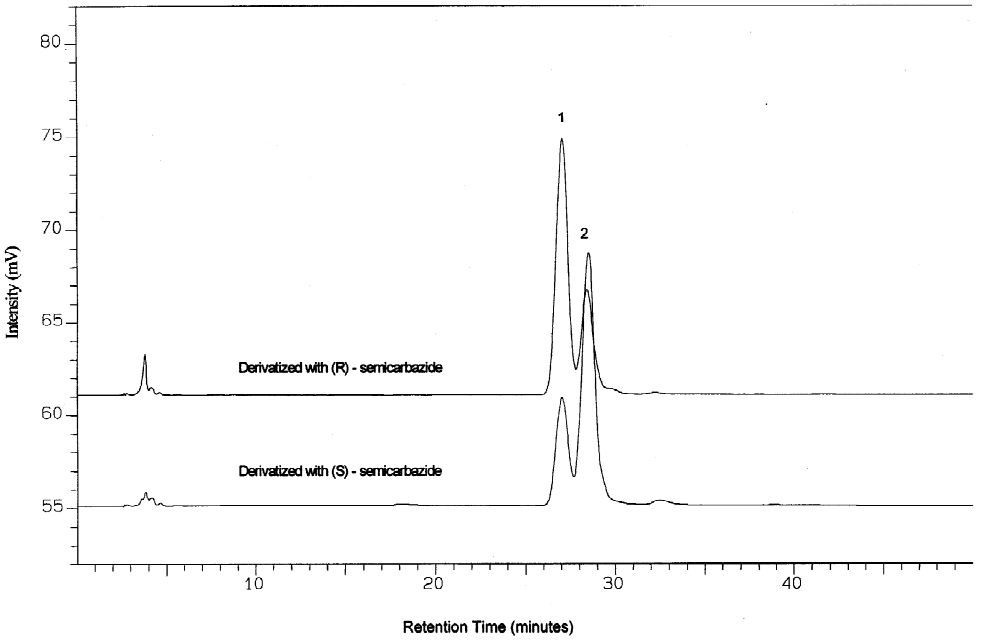

Fig. 6. HPLC separation of SCH 201781 semicarbazones. Achiral separation conditions: MetaChem Hypersil BDS column, 5 μm, 25 cm×4.6 mm I.D.; mobile phase, methanol–0.01*M* phosphate buffer at pH 7.5 (21:29 v/v); flow rate of 1.0 ml/min; column temperature of 30°C; detection in UV–Vis at 310 nm; injection volume, 10μl of 0.4 mg/ml SCH 201781 enantiomeric mixture, Peaks: 1, (*S*,*R*)/(*R*,*S*)-semicarbazone; 2, (*S*,*S*)/(*R*,*R*)-semicarbazone.

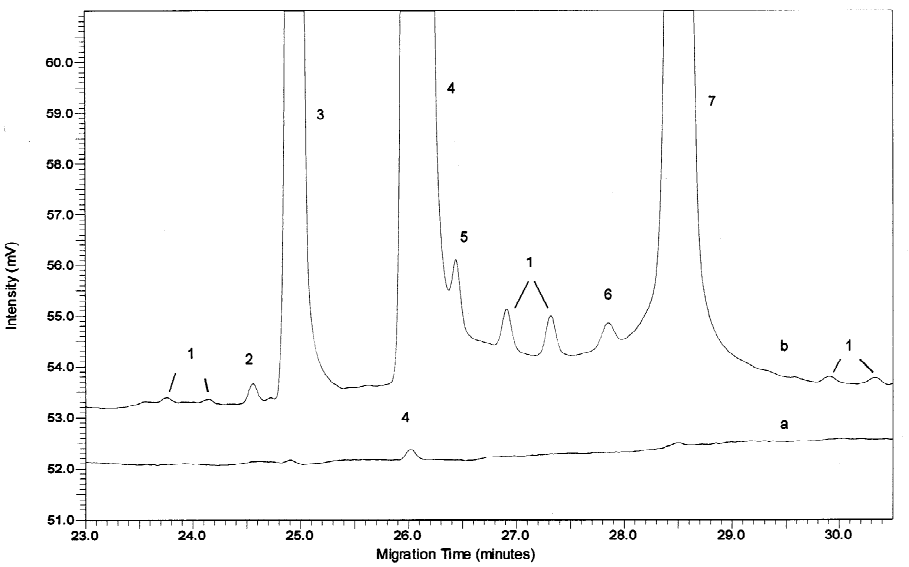

Fig. 7. Representative electropherograms of a LOQ solution and a sample solution. Background electrolyte: 50 m*M* citrate buffer at pH 2.50 with 20 m*M* SBE-β-CD and 5 m*M* β-CD; PVA coated capillary, 56 cm effective length ×50 um I.D.; applied voltage, (−) 20 kV, 40 μA; injection, 250 mbar s (hydrodynamic mode) of (a) LOQ solution, 2 μg/ml SCH 2101781, (b) sample solution (containing 0.5% enantiomer), 2 mg/ml SCH 201781; detection, 200 nm. Peaks: 1, process impurities; 2, (*R*,*R*)-aminal I; 3, (*S*,*S*)-aminal I; 4, (*S*,*R*)-aminal II; 5, (*R*,*S*)-aminal II; 6, (*R*)-hydrate; 7, (*S*)-hydrate.

Table 4
Comparison of quantitative parameters for determination of enantiomers using CZE and HPLC

Parameter	CZE[a]	HPLC[b]
Replicate injections, mean±% RSD ($n=5$)	2.7±0.7	1.3±3.9
Replicate sample assays for enantiomer content, mean±% RSD ($n=3$)	1.1±1.6	1.3±5.8
Recovery of enantiomer at levels of		
0.1%	100	–[c]
1.0%	100	–[c]
5.0%	98	–[c]
Linearity,		
range (% enantiomer content)	0.1–8.0	–[c]
R^2	0.999	–[c]
Limit of quantitation ($S/N=10$)	0.1%	0.5%
Limit of detection ($S/N=3$)	0.03%	0.2%

[a] CZE conditions: background electrolyte, 50 m*M* citrate buffer at pH 2.50 with 25 m*M* SBE-β-CD. fused-silica capillary, 72 cm effective length×75 μm I.D.; applied voltage, (−) 15 kV; injection, 250 mbar s (hydrodynamic mode) of 2 mg/ml of SCH 201781; detection, 200 nm.

[b] HPLC conditions: MetaChem Hypersil BDS column, 5 μm, 25 cm×4.6 mm I.D.; mobile phase, methanol–0.01*M* phosphate buffer at pH 7.5 (21:29,v/v); flow rate of 1.0 ml/min; column temperature of 30°C; UV detection at 310 nm; injection volume, 10 μl of 0.4 mg/ml of SCH 201781.

[c] Not tested.

measured for the second peak, (*S*,*R*)-aminal II. A representative electropherogram of a LOQ solution (0.1%) and a sample solution of SCH 201781 containing 0.5% of enantiomer are shown in Fig. 7. The HPLC method was limited to an upper concentration of 0.4 mg/ml, above which the resolution drastically decreases because of peak distortion.

Table 5
Comparison of SCH 201781 enantiomer content using CZE and HPLC methods

Batch	% Enantiomer	
	CZE[a]	HPLC[a]
UB-3A	1.2	1.2
UB-3B	1.3	1.3
UB-3C	1.8	1.8
UB-3D	2.0	2.0
UC-01	0.5	0.5
UC-02	1.3	1.3

[a] CZE conditions and HPLC conditions refer to Table 4.

3.3.2. Comparison batch data using CZE and HPLC

Six batches of SCH 201781 were analyzed for enantiomer content using both the CZE and HPLC methods. The results (Table 5) show an excellent correlation for the amounts of enantiomer using both procedures.

4. Conclusion

This paper describes CZE and precolumn derivatization HPLC methods for enantiomeric separations of a complex molecule. The utility of the CZE method for routine analysis was demonstrated by precision, recovery, linearity and sensitivity data. Comparable results for enantiomer content on test samples were obtained using both methods. The CZE method provides better sensitivity (LOQ=0.1%) than the HPLC method (LOQ=0.5%) and more precise (RSD=1.6%) than HPLC (RSD=5.8%). In addition, the CZE method is superior to the HPLC method because it does not require precolumn derivatization and it provides more information about the various forms of SCH 201781 in solution.

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