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Separation and determination of cefotaxime enantiomers in injections by capillary zone electrophoresis

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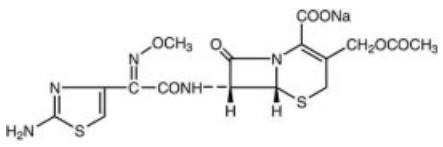
Cefotaxime enantiomers have specific effects on Gram-negative bacteria. For quality control of cefotaxime it was necessary to establish a method for enantioseparation by capillary zone electrophoresis (CZE) using cyclodextrin (CD) as a chiral selector. The effects of various parameters on enantioseparation were studied. A fused silica capillary (40 cm effective length $\times$ 75 μm ID) was used. The cefotaxime enantiomers were separated on the baseline under conditions of 0.5 mmol/L CM-β-CD, 75 mmol/L NaH_2PO_4 buffer at pH 7.0 using UV detection at 280 nm. Applied voltage and capillary temperature were 20 kV and 25 °C, respectively. Under these conditions for enantioseparation, linear calibration curves were obtained in the range 2 $\sim$ 160 μg/mL. The limit of detection for both isomers was less than 0.5 μg/mL. The method was used for analysis of pharmaceutical preparations (dosage forms) of cefotaxime from various factories. A simple and specific CZE method was successfully demonstrated for the separation of cefotaxime enantiomers. The enantioseparation method should be established and this method should be used to control the quality of cefotaxime.

1. Introduction

Cefotaxime sodium, sodium [6R, 7R]-3-[(acetyloxy)-methyl]-[[(2Z)-(2-amine-4-thiazolyl)(methoxyimino)-acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4,2,0]oct-2-ene-2-carboxylate, is a semisynthetic-β-lactam antibiotic, which belongs to the class of third generation cephalosporin antibiotics. It has a chiral carbon atom and enantiomers exist. Its various formulations have wide clinical use because of its broad anti-biogram, anti-bacterial activity, and acid and enzyme resistance.



Clinical pharmacology studies have shown that on administration of a single 500 mg or 1 g dose of cefotaxime to normal volunteers, mean peak serum concentrations of 11.7 and 20.5 μg/mL respectively were attained within 30 min and the level then declined with an elimination half-life of approximately 1 h. Cefotaxime may cause side effects including diarrhea, stomach ache, dyspepsia and vomiting depending on quality and the different enantiomers. Cephalosporin antibiotics are widely used clinically. In order to improve the bioactivity of cephalosporin and control the quality of cephalosporin (Van Hoogdalem et al. 1989), an efficient analytical method should be developed to determine these drugs both in pharmaceutical formulations and in biological media. Liquid chromatography (Gonzalez-Corbella 1994; Scanes et al. 2001; Galanti et al. 1996; Liang et al. 1994) and capillary electrophoresis (Mrestani et al. 1997; Choi et al. 1997; Castaneda Penalvo et al.1997; Honda et al. 1992; Li et al. 1999; Pajchel et al. 2001) have been used extensively for the separation and assay of cephalosporin in formulations and biological samples. Because cefotaxime is widely used to prevent the infection in operations and as therapy for infection, and the two enantiomers are known to have different bioactivities, and as no paper has yet reported the enantioseparation of cefotaxime by CE or other methods, developing a good method for the separation of cefotaxime enantiomers is necessary.

In recent years, CE has become a powerful technique for enantioseparation (Koppenhoefer et al. 2000) that is successfully utilized for separation of optical isomers as discussed in many review papers (Amini 2001; Fanali 1997). A number of pharmaceutical drugs have been chirally resolved employing various chiral additives. In most cases, cyclodextrins (CDs) and their derivatives have been used as chiral selectors in CE because of their applicability to the separation of a wide range of structurally different compounds (Gübitz et al. 1997; Maier et al. 2001; Fanali et al. 1997; Quang et al. 1995). For the cephalosporins studied it has been reported that cephalosporin preparations and biological samples were enantioseparated and detected by CE methods (Wang et al. 2002, 2003, 2004). This can provide information relevant to diagnosis and therapy in clinical practice.

The aim of the present work was to develop a rapid and efficient method for separation of cefotaxime enantiomers

by capillary zone electrophoresis using CD as a chiral selector for quality control of cefotaxime. This enantioseparation method was to be used to monitor the production of cefotaxime pharmaceuticals and control the quality of cefotaxime in clinical use.

2. Investigations and results

2.1. Method development and optimization

A range of α-CD, β-CD, γ-CD, HP-β-CD and Me-β-CD have been explored for enantiomeric separation, but no resolution (Rs) data were available. Good baseline resolution of cefotaxime enantiomers was obtained using CM-β-CD as the additive in this experiment. Figure 1a shows the Rs of enantioseparation by α-CD, β-CD, γ-CD, HP-β-CD, Me-β-CD and CM-β-CD. The electropherogram of enantioseparation by CM-β-CD is shown in Fig. 1b.

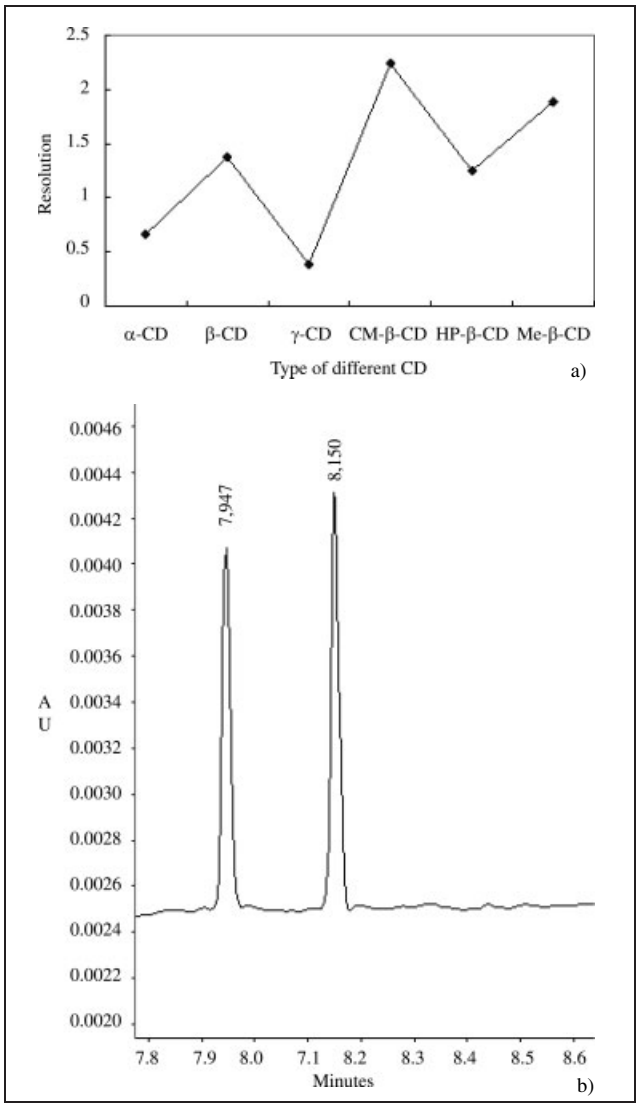


Fig. 1: a) Effect of different CDs on the resolution of the cefotaxime enantiomers
Capillary: fused silica (40 cm × 75 μm I.D.); applied voltage: 20 kV, detection: UV absorption at 280 nm, buffer: 75 mmol/L NaH_2PO_4, pH = 7.0, 25 °C
b) Electropherograms of cefotaxime enantiomers standard
Capillary: fused silica (40 cm × 75 μm I.D.); applied voltage: 20 kV, detection: UV absorption at 280 nm, buffer: 75 mmol/L NaH_2PO_4, pH = 7.0, 25 °C

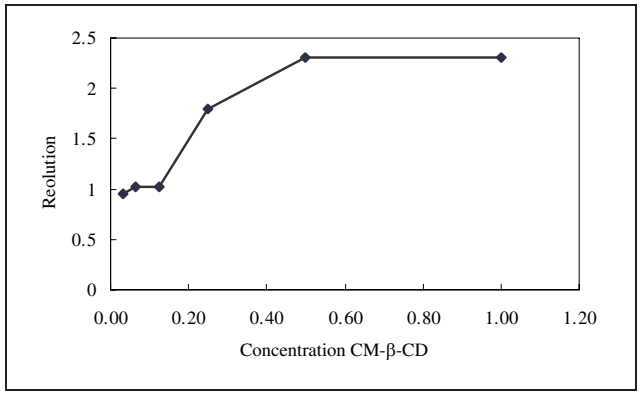


Fig. 2: Effect of concentration of CM-β-CD on the resolution of the cefotaxime enantiomers
Capillary: fused silica (40 cm × 75 μm I.D.); applied voltage: 20 kV, detection: UV absorption at 280 nm, buffer: 75 mmol/L NaH_2PO_4, pH = 7.0, 25 °C

The effects on separation of CM-β-CD concentrations between 0.03 and 1 mmol/L in phosphate buffer were studied. Over the range of CM-β-CD concentration from 0 to 1 mmol/L, the resolution for cefotaxime enantiomers increased as shown in Fig. 2. As expected, the migration time increased as the concentration of CM-β-CD in the running buffer increased, as more stable analyte-cyclodextrin complexes were formed. The cefotaxime enantiomers were very well separated at 0.5 mmol/L CM-β-CD, with Rs over 2. 0.5 mmol/L CM-β-CD was thus considered as the optimal concentration for the separation of cefotaxime enantiomers with a reasonable migration time.

Keeping the other conditions constant, different buffer concentrations (15, 37.5, 50, 70 and 100 mmol/L) of NaH_2PO_4 were studied. Because of the current involved, the problem of heat generation/dissipation should be noted. To prevent the generation of too much Joule heat, and to obtain best separation, 75 mmol/L NaH_2PO_4 buffer was chosen.

Optimization was performed with 75 mmol/L phosphate buffer and 0.5 mmol/L CM-β-CD, at 20 kV voltage and a temperature of 25 °C. pH was varied between 3 and 9 in steps of 1. The migration time of cefotaxime enantiomers in CZE decreased with increased buffer pH in the range 3 to 7, while in the pH range 7 to 9 the migration time was increased. Where the cefotaxime enantiomers had negative electrophoretic mobility, this was probably due to the characteristic highly acidic 3-thiomethyl group attached to the C-3 position of the cephem structure in the heterocyclic system. Separation of cefotaxime enantiomers could be achieved at pH 7.0. Results are shown in Fig. 3.

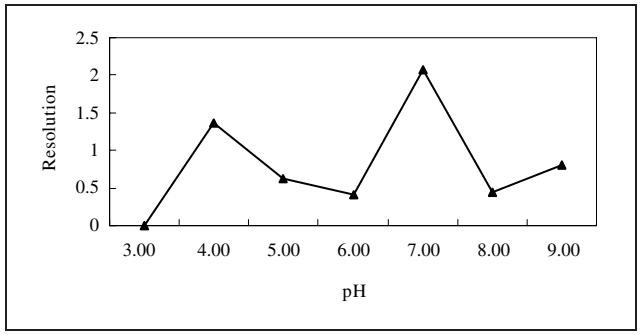


Fig. 3: Effect of pH on the resolution
Capillary: fused silica (40 cm × 75 μm I.D.); applied voltage: 20 kV, detection: UV absorption at 280 nm, buffer: 75 mmol/L NaH_2PO_4, 25 °C

Four voltages (10, 15, 20, 25 kV) were studied. The optimal voltage was set at 20 kV, which gave the shortest migration time and generated acceptable current.
The optimal electrophoretic parameters included: 0.5 mmol/L CM-β-CD, 75 mmol/L NaH_2PO_4 buffer at pH 7.0, 20 kV and a capillary temperature of 25 °C. Figure 1b shows a typical electropherogram of a cefotaxime standard sample. Because single cefotaxime enantiomers could not be obtained, we considered that the cefotaxime enantiomer I (R-(+)-) corresponded to the first peak, while cefotaxime enantiomer II (S-(−)-)corresponded to the second peak.

2.2. Method validation

Linear regression analysis for cefotaxime enantiomers was made by plotting the peak area to migration time ratio (y) versus the analytical concentration of cefotaxime enantiomer standards (x, μg/mL). The following equations were obtained (concentration range: 2.0 ∼ 160.0 μg/mL for cefotaxime enantiomers, respectively):

R-(+)-cefotaxime: $y = 24763.50x + 5973.7$ $r = 0.9981$ (1)

S-(−)- cefotaxime: $y = 28834.52x + 6045.7$ $r = 0.9990$ (2)

The linearity of the relationship between peak area and concentration was demonstrated by the correlation coefficients (r) for the regression lines for both cefotaxime enantiomers.
A signal-to-noise ratio of approximately 3 is considered acceptable for estimation of limit of detection. The LOD of the enantiomers was found to be 0.4 μg/mL and 0.5 μg/mL, respectively. The limit of quantification (LOQ) is the lowest concentration of a compound that can be quantified with acceptable precision and accuracy. A concentration with a signal-to-noise ratio of 10 is typically regarded as the LOQ level. The LOQ for the enantiomers was found to be 0.8 μg/mL and 0.86 μg/mL, respectively.
Repeatability was determined by making six replicate injections of a standard solution at limit of quantitation (LOQ) level and at 1% level. The relative standard deviation (R.S.D.) values for migration time, correct peak area and corrected peak area ratio are given in Table 1.
The precision of the method was determined by measuring repeatability (n = 6) and intermediate precision (n =3 days) for both enantiomers at four different concentration levels ranging from 2.0 to 160.0 μg/mL. The mean values for intra-day and inter-day precision were 6.4 % and 7.6% for R-(+)- cefotaxime, and 6.3% and 7.4% for S-(−)-cefotaxime, respectively (see Table 2).
Samples were independently prepared in triplicate by spiking a cefotaxime standard solution at LOQ, 0.8%, 1.0% and 1.2% levels of the standard drug. The percentage recoveries were calculated from the slope and intercept obtained from the standard curve. The percentage recovery ranged from 99.6 to 100.5% as shown in Table 2.
There was no significant change either in the peak area ratios or in enantiomeric composition confirming that no degradation or inter conversion of the enantiomers took place for at least 24 h on the room temperature.
Samples solutions were filtered through 0.45 μm syringe filters before injection. The results of the analysis of various batch samples of cefotaxime are shown in Table 3.
Applications of the method to the assay of cefotaxime enantiomers in pharmaceutical dosage forms were studied. Table 3 shows the results obtained for the analysis of cefotaxime for injection (contained cefotaxime 0.5 g per vial). The concentration of cefotaxime enantiomers was determined in products from different factories by the CE method. The results showed that while the preparations had passed quality control for rac-cefotaxime, the concentrations of cefotaxime enantiomers by CE were very different for products from the different factories. Quality control of cefotaxime enantiomers is thus necessary.

Table 1: Precision data

	LOQ Level (RSD %)		1% Level (RSD %)	
	R-(+)-	S-(−)-	R-(+)-	S-(−)-
Repeatability[a]				
Migration time	0.62	0.72	0.18	0.23
Corrected peak area	6.13	7.54	2.01	1.65
Intra-day precision				
Migration time	0.55	0.63	0.67	0.76
Corrected peak area	5.82	6.01	3.28	4.98
Inter-day precision				
Migration time	1.22	1.35	1.08	1.46
Corrected peak area	5.06	4.68	2.52	2.32

[a] n = 6 determinations

Table 3: Results for quantification of pharmaceutical (cefotaxime for injection) of five factories

Factory	Batch No.	Determined (mg/mL)		RSD (%)	
		R-(+)-	S-(−)-	R-(+)-	S-(−)-
1	06031745123	0.43	0.34	2.86	4.53
2	B070633412	0.52	0.40	3.12	4.28
3	0702030	0.36	0.42	3.13	4.56
4	0610203	0.61	0.38	2.72	2.86
5	0612113214	0.45	0.56	2.96	3.23

Table 2: Recovery and accuracy of cefotaxime enantiomers

Number[a]	Added g/mL	RSD% of Detected g/mL		CV%				Recovery %	
		R-(+)-	S-(−)-	Intra-day		Inter-day		R-(+)-	S-(−)-
				R-(+)-	S-(−)-	R-(+)-	S-(−)-		
6	2	8.5	4.2	7.2	7.2	8.8	7.7	98.5%	99.8%
6	40	5.1	4.8	6.7	5.9	6.6	8.3	97.8%	102.3%
6	80	4.6	6.2	5.8	7.1	6.9	8.1	102.8%	98.2%
6	160	5.3	4.1	5.8	4.8	8.0	5.5	99.2%	101.6%
Av		5.9	4.8	6.4	6.3	7.6	7.4	99.6%	100.5%

[a] Number of analyses

3. Discussion

A simple and specific CZE method has been successfully demonstrated for the separation of cefotaxime enantiomers. Six CDs were tested as chiral additives, but only CM-β-CD offered satisfactory separation of cefotaxime enantiomers. Under optimized conditions, the method has been applied to determination of the concentration of cefotaxime enantiomers in pharmaceutical preparations. It was found that different concentrations of cefotaxime enantiomers were obtained from different factories using this method. Thus, this enantioseparation method should be established and used for quality control of cefotaxime.

4 Experimental

4.1. Chemicals and reagents

Cefotaxime enantiomer standards were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). α-CD, β-CD, γ-CD, HP-β-CD and Me-β-CD was purchased from Sigma Chemical Co. (St. Louis, MO, USA). CM-β-CD were purchased from Merck & Co., Inc. (New Jersey, USA). Methanol was of HPLC grade (Siyou, Tianjin, China). Sodium dihydrogen phosphate was obtained from Hongxing (Beijing, China). 0.45 μm nylon filters were purchased from Kaide (Tianjin, China). Cefotaxime for injections was purchased from Lanzhou Pharmacy (Gansu, China). Water was distilled, deionized and filtered with 0.45 μm nylon membranes in our laboratory.
Cefotaxime for injection (0.5 g per vial) were obtained from five factories in China.

4.2. Apparatus

CE was performed on a Beckman P/ACE 5000 capillary electrophoresis system with Beckman P/ACE software station 1.0. UV detection (Beckman, USA) was at 280 nm. A fused-silica capillary (40 cm effective length × 75 μm I.D.) (Beckman, USA) was employed.

4.3. Assay preparation

Cefotaxime enantiomer standards were dissolved in water to obtain stock standards of cefotaxime (2 mg/mL). Working solutions were prepared by dilution of the stock solutions with water.
Cefotaxime for injection solutions were prepared as follows: A portion of powder was weighed accurately, equivalent to about 20 mg, and transferred to a 10-ml volumetric flask. Deionized water was added to dissolve the powder. It was then diluted to volume with deionized water. The injection solutions were assayed by CE.
All samples and buffers were both filtered through 0.45 μm syringe filters and degassed ultrasonically before assaying. All solutions were stored at 4 °C.

4.4. Procedures

The capillary was conditioned at the beginning of the day with 0.1 mol/L NaOH for 5 min and followed by a water wash for 2 min. The running buffer was phosphate buffer (75 mmol/L NaH_2PO_4, pH 7.0) containing CDs. Before every analysis, the capillary was washed for 5 min with running buffer. After several runs, the following wash sequence was performed: 2 min with 0.1 mol/L NaOH, 2 min with deionized water and 3 min with running buffer. Electrophoresis was performed at a voltage of 20 kV and a temperature of 25 °C using UV detection at 280 nm; the average current was about 136 μA. Samples were injected in pressure mode for 6 s at 0.5 p.s.i. The separation was carried out from the positive to the negative electrode.

4.5. Method development and optimization

4.5.1. Type and concentration of cyclodextrin

Six different CDs: α-CD, β-CD, γ-CD, HP-β-CD, Me-β-CD and CM-β-CD were compared as chiral additives for the separation of cefotaxime enantiomers when the other conditions (75 mmol/L NaH_2PO_4, pH 7.0, 20 kV and 25 °C) were kept constant. The type of cyclodextrin affected resolution significantly in this study. Since the cefotaxime enantiomers have the same mass-to-charge ratio, when inclusion complexes of cefotaxime with chiral cyclodextrin were formed, enantioseparation of the two enantiomers was due to the delay in migration of the inclusion complexes.

4.5.2. Analytical voltage

Both the electroosmotic and electrophoreic velocities are directly proportional to the field strength, so that using the highest possible voltages will result in the shortest time for separation. The shortest separation time will give the highest efficiency since diffusion is the most important feature contributing to band broadening. The limiting factor here is Joule heating.

4.6. Method validation

Validation of the enantioseparation assay method was performed according to standard operating procedures for bioavailability and bioequivalence. Under the optimal separation conditions obtained, the parameters of the validation procedure in the experiments included: linearity and range, limit of detection and limit of quantitation, precision, accuracy and selectivity of the bioanalytical method.

4.6.1. Linearity and range for R-(+)- and S-(−)- cefotaxime

Calibration curves were established using concentration linear regression for cefotaxime standard solutions, which were linear in the range from 2.0 to 160.0 μg/mL of cefotaxime enantiomers.

4.6.2. Precision for R-(+)- and S-(−)-cefotaxime

The precision of the method was determined in terms of repeatability, and intra-day and inter-day precision (intermediate precision) of migration times and corrected peak area ratios. Since the area of a peak is dependent on its electrophoretic mobility, the corrected area, i.e. the ratio of peak area and migration time which remains constant, is considered in capillary electrophoresis.

4.6.3. Accuracy

The accuracy of the method was established by performing recovery experiments.

4.6.4. Stability

The stability of the sample in solution was evaluated by injecting the sample immediately after preparation and then reanalysing after storing it on the bench-top for 24 h.

4.6.5. Quantification of cefotaxime enantiomers

Samples of about 5 batches of cefotaxime injection from five factories in China were analyzed for content of R-(+)- and S-(−)-isomers. About 10 mg of each test sample was transferred into a separate 10 mL volumetric flask.

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