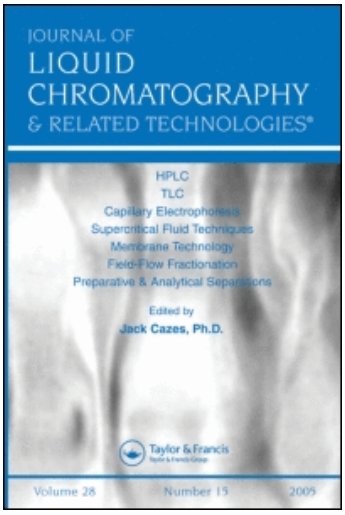

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Capillary Electrophoretic Separation and Quantitation of Ractopamine Stereoisomers Using Cyclodextrins as Chiral Additives

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Greenfield, Indiana, USA

ABSTRACT

Separation of four stereoisomers of ractopamine was conducted by capillary electrophoresis (CE) using a variety of neutral and charged cyclodextrins (CDs) as chiral selectors added into the running buffer. Among the CDs used in the screening study, only two CDs, sulfated α-CD (S-α-CD) and heptakis-(2,3-diacetyl-6-sulfato)-β-CD (HDAS-β-CD), gave the baseline separation of each isomer. The separation conditions such as running buffer pH, concentration of the CD, and separation voltage were further optimized using S-α-CD, which provided the favorable migration order for quantitative studies. The method was validated and applied to the determination of the trace amounts of the less bioactive isomers (*SR*-, *RS*-, and *SS*-ractopamine). 0.2 ~ 0.5% (wt.%) of the less

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bioactive isomers were able to be determined using this method. The method was also able to quantitate ractopamine isomers extracted from the feed premix for swine. The recovery of *RR*-ractopamine from the feed premix was $92.6 \pm 4.6\%$.

Key Words: Ractopamine; Capillary electrophoresis; Cyclodextrins; Multiple chiral centers.

INTRODUCTION

In the pharmaceutical industry, chirality of drug candidates is one of the important concerns involved in the drug development process.[1] Whether a single isomer or a mixture of isomers, chiral drug candidates require more analytical information than achiral ones, including chiral purity and stability of candidates.[1] In order to obtain such information, the separation and quantitation of enantiomers are essential. However, direct chiral separation has been a great challenge for analytical chemists during the past few decades. When compounds of interest have multiple chiral centers, the separation of stereoisomers is even more difficult because the chiral centers have to be differentiated simultaneously in the chiral media.

Since the first report in 1985,[2] capillary electrophoresis (CE) rapidly became a versatile technique for chiral separations and many review articles have been published.[3–6] The most commonly used chiral selectors in CE are cyclodextrins (CDs) and their derivatives. Recently, charged CDs have been introduced[7,8] and found to be very useful for the chiral separation of many compounds including basic drugs.[9–15] However, only a limited number of compounds with multiple chiral centers has been previously studied using charged CDs.[16–18]

The compound analyzed in this study is ractopamine hydrochloride (Fig. 1). Ractopamine, one of the phenethanolamine β-adrenergic agonists, has been approved by FDA as feed additive for swine.[19] It has been shown that ractopamine reduces fat and increases muscle mass, while improving growth and carcass performance, and feed utilization efficiency

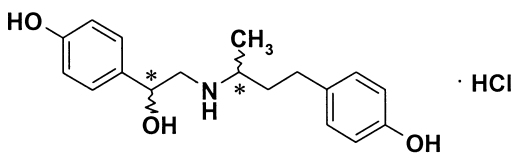

Figure 1. Structure of ractopamine HCl.

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when fed to swine.[20,21] Ractopamine has two chiral centers and is currently commercialized as a mixture of four stereoisomers in approximately equal proportions, although studies have shown that the *RR* isomer is responsible for a majority of the leanness-enhancing effects of ractopamine in rats.[22] This paper presents the separation of four stereoisomers of ractopamine by CE, using both neutral and charged CDs as chiral selectors. The optimization of the separation conditions and the application of the method to the quantitative analysis, such as determination of the trace amounts of less bioactive isomers and determination of each isomer in the feed premix for swine are also reported.

EXPERIMENTAL

Chemicals

β-Cyclodextrin (β-CD) and heptakis-(2,6-di-*o*-methyl)-β-CD (HDM-β-CD) were purchased from Sigma (St. Louis, MO). Heptakis-(2,3-diacetyl-6-sulfato)-β-CD (HDAS-β-CD), heptakis-(2,3-dimethyl-6-sulfato)-β-CD (HDMS-β-CD), and heptakis-6-sulfato-β-CD (HS-β-CD) were obtained from Regis Technologies (Morton Grove, IL). Hydroxypropyl-α-CD [HP-α-CD, degree of substitution (DS) = 3.5], hydroxypropyl-β-CD (HP-β-CD, DS = 5.3), hydroxypropyl-γ-CD (HP-γ-CD, DS = 6.4), sulfated α-CD (S-α-CD, DS = 12), sulfated β-CD (S-β-CD, DS = 4 and 14), and quaternary ammonium-β-CD (QA-β-CD, DS = 3.5) were from Cerestar (Hammond, IN). γ-Cyclodextrin was from Beckman Instruments (Fullerton, CA). Racemic ractopamine hydrochloride and the feed premix for swine (Paylean®) were obtained from Lilly Research Laboratories (Indianapolis, IN). Four stereoisomers of ractopamine, *RR*-, *RS*-, *SR*-, and *SS*-ractopamine, were synthesized in USDA-ARS Biosciences Research Laboratory (Fargo, ND). All other chemicals were analytical grade and used as received. The average molecular weight values, which were provided by the manufacturers, were used to make up the solutions of randomly substituted CDs.

Buffer and Sample Preparation

The buffer solutions with desired pHs were prepared by dissolving the appropriate salt and adjusting pH with acid or base. The running buffers were prepared by adding desired amounts of CDs into each buffer solution. For the quantitative studies, three different sample solutions were prepared by mixing four of each isomer with various ratios: (1) $RR:SR:RS:SS = 88:4:4:4$; (2) $RR:SR:RS:SS = 90:5:2:3$; and (3) $RR:SR:RS:SS = 86:$

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3 : 5 : 6. The total amount of the isomers in each sample solution was 0.05 mg/mL. All the solutions were filtered through 0.2 μm syringe filters (Acrodisc® CR PTFE) from German Sciences (Ann Arbor, MI) before use.

Sample Preparation for Analysis of Ractopamine in Feed Premix

Fifty milliliters of methanol was added into 5 g of the feed premix for swine, which contains 2% of ractopamine, and the mixture was shaken by the gyratory shaker (New Brunswick Scientific, Edison, NJ) for 2 hours at the speed of 300 rpm. The sample solution was then diluted 10 times with Milli-Q water and filtered through 0.2 μm syringe filters prior to injection.

Capillary Electrophoresis

All CE experiments were carried out on a P/ACE MDQ capillary electrophoresis instrument (Beckman Instruments, Fullerton, CA), using a 50 μm i.d. × 60 cm (50 cm effective length) fused silica capillary (Polymicro Technologies, Phoenix, AZ) at 20°C. The UV detection wavelength was 214 nm. The newly prepared capillary was treated for 10 min with 0.1 M NaOH, followed by rinsing with water for 10 min, then, finally the desired buffer solution for 15 min at 20 psi. Between runs, the capillary was washed with the running buffer for 5 min. Samples were injected by applying pressure of 0.5 psi for 5 s. Mesityl oxide (Sigma) was used as an electroosmotic flow (EOF) marker. The separation voltage was +20 kV for all the experiments, except for the study of separation voltage effects where the voltage was changed from +10 kV to +30 kV. The attempt to employ the reverse polarity for the analyses using the negatively charged CDs failed due to the insufficient separation. All the data are given as average values of triplicate CE runs unless otherwise noted.

RESULTS AND DISCUSSION

Method Development

Chiral Selector Screening

In this study, 13 different CDs were screened to investigate the possibilities of CDs to separate four stereoisomers of ractopamine. The results, using the optimum concentration of each CD at pH 2.5, are summarized in Table 1. Among them, only two CDs, S-α-CD and HDAS-β-CD, could achieve base-

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Table 1. Screening of the CDs as the chiral selectors for separation of ractopamine stereoisomers.

Selector	Charge	Conc. (mM)	Elution order 1	2	3	4
β-CD	Neutral	20	$RS+SR$		$RR+SS$	
γ-CD	**Neutral**	**20**	***RS***	***SR***	***RR***	***SS***
			0.1[a]	1.2[a]	0.7[a]	
HP-β-CD	Neutral	20	RS	SR	$RR+SS$	
HP-α-CD	Neutral	20	$RS+SR$		$RR+SS$	
HP-γ-CD	Neutral	20	$RS+SR+RR$		SS	
HDM-β-CD	Neutral	20	RR	$RS+SR+SS$		
QA-β-CD	Positive	20	$RS+SR+RR+SS$			
S-α-CD	**Negative**	**0.5**	***SR***	***RS***	***SS***	***RR***
(DS = 12)			1.8[a]	2.1[a]	2.7[a]	
S-β-CD (DS = 14)	Negative	0.1	$RS+SR$		SS	RR
S-β-CD (DS = 4)	Negative	0.1	$RS+SR$		$RR+SS$	
HS-β-CD	Negative	0.2	$RS+SR$		SS	RR
HDMS-β-CD	Negative	0.5	$RS+SR$		$RR+SS$	
HDAS-β-CD	**Negative**	**0.4**	***SR***	***RS***	***RR***	***SS***
			1.8[a]	3.1[a]	7.6[a]	

Note: pH: 2.5, separation voltage: +20 kV, capillary temperature: 20°C.
[a]R_s values between the adjacent peaks.

line separation of four isomers. γ-Cyclodextrin could separate four isomers but had insufficient R_s values (see the R_s values in Table 1). All other CDs showed no or only partial separation of some of the four isomers.

Both S-α-CD and HDAS-β-CD are negatively charged, indicating that the negative charge has preferable effects on separation of ractopamine stereoisomers. One of the effects is the improved stability of the diastereomeric complexes due to the ionic attraction between the negatively charged CDs and ractopamine, which is positively charged at pH 2.5 (ractopamine pK_1 ~8.9, pK_2 ~9.2, pK_3 ~11.0). Another effect would be the opposite electrophoretic mobilities of the CD/ractopamine complex and free ractopamine, which allow the prolonged stay of the complex in the capillary, and consequently, better separation. However, having a negative charge may not be enough for separation of four stereoisomers, since the other negatively charged CDs failed to achieve the baseline separation of four isomers. Other types of stereospecific interactions may be involved in the complete separation.

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Based on the results shown above, S-α-CD was chosen as a selector for further investigation of ractopamine separation due to the favorable migration order of the isomers, i.e., the less bioactive isomers *RS, SR*, and *SS*-ractopamine were eluted prior to *RR*-ractopamine. This migration order can prevent interference by the *RR* peak tailing due to the large sample loading when the trace levels of less bioactive isomers need to be determined.

Buffer pH

The pH of the running buffer has significant effects on separation by CE since the charges of analytes and/or chiral selectors as well as EOF are greatly dependent on pH. Sulfated α-CD is a strong electrolyte and maintains the negative charges in the entire pH range used in this study (pH 2.5 ~ 11.0). Ractopamine has three ionizable groups, the secondary amine group ($pK_a = 8.85$), the *p*-hydroxymethinyl-phenol segment ($pK_a = 9.21$), and the alkylphenol moiety ($pK_a = 11.01$). It has been shown that all three ionizable groups are severely overlapped in aqueous solution and yielded a single inflection point at 9.42 by conventional potentiometry. In Fig. 2, the

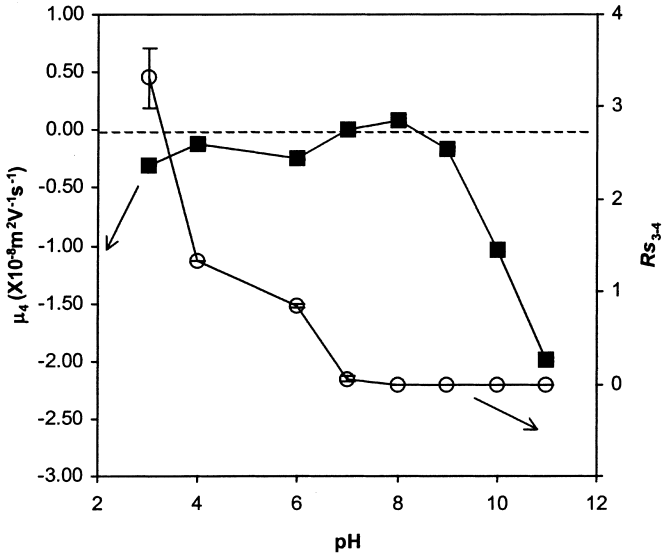

Figure 2. Effect of running buffer pH on separation of ractopamine stereoisomers. *Key:* ■, relative mobility of desired peak, *RR*; ○, R_s between third and fourth eluted peaks, *SS* and *RR*. Concentration of S-α-CD: 1 mM. Separation voltage: +20 kV. Capillary temperature: 20°C.

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plots of effective mobility of the bioactive peak (*RR*) and resolution between third and forth eluted peaks (*SS* and *RR*, respectively) vs. pH are shown using 1 mM S-α-CD. At pH 7.0 or lower, ractopamine has the slightly negative mobility, i.e., the net negative charge. In this pH range, ractopamine itself is positively charged. Thus, this result implies the complexation between ractopamine and S-α-CD. The complexation is also proven by the fact that the separation of the ractopamine isomers (at least partially) was observed in this pH range. At pH 8.0, where ractopamine should be still positively charged to some extent, the mobility became positive, indicating that the complexation is weaker than that at a lower pH. This is probably because the secondary amine group is no longer fully protonated at pH 8.0. At pH 9.0, the mobility went back to negative again, then rapidly became more negative at higher pH due to the deprotonation of one or both phenol groups. Since ractopamine is negatively charged, the complexation with S-α-CD is less probable, although it is not impossible, as no separation was observed in this pH region.

At pH 3.0, the baseline separation of all four isomers was achieved. However, the peaks showed severe tailing and the migration time was too long. At pH 6.0, the peak tailing was minimized although the resolution was compromised by the shorter analysis time under the given conditions. For the rest of study, pH 6.0 was used and the separation was further optimized to obtain better separation.

Concentration of Sulfated α-Cyclodextrins

The separation of ractopamine stereoisomers is also influenced by the concentration of S-α-CD in the running buffer. In Fig. 3, the selector concentration effects on the effective mobility and resolution are shown. The mobility had positive values up to ~0.5 mM S-α-CD, became negative as the concentration increased, and then leveled off around 5 mM and higher. This implies that ractopamine forms the complex with S-α-CD only weakly at a lower S-α-CD concentration, maintaining the net positive charge, while at a higher concentration the complex is strong enough to give ractopamine the net negative charge. The resolution improved as the concentration increased corresponding to the increased stability of the complex. However the resolution still increased even at higher than 5 mM, at which the mobility was leveled off. This can be attributed to the smaller EOF due to the larger ionic strength of the running buffer at the higher concentration of S-α-CD, which may increase the resolution.

According to the results, 3 mM or higher concentration of S-α-CD can offer the baseline separation of four isomers. The appropriate concentration of S-α-CD should be chosen depending on the types of application. For example, when the compound of interest is consisted of nearly equal amount of each

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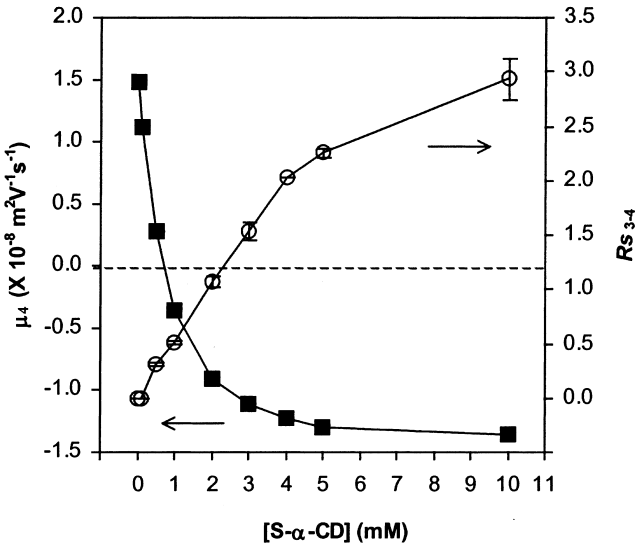

Figure 3. Effect of S-α-CD concentration on separation of ractopamine stereoisomers. *Key:* ■, relative mobility of desired peak, *RR*; ○, R_s between third and fourth eluted peaks, *SS* and *RR*. Running buffer: 20 mM phosphate (pH 6.0), separation voltage: +20 kV, capillary temperature: 20°C.

isomer, the baseline separation is sufficient enough for the quantitative analysis of each isomer. In this case only ~3 mM of S-α-CD would be necessary. Meanwhile, 5 mM or higher concentration would be required for determination of the trace amounts ($\leq 1\%$) of isomeric impurities where the larger resolution is preferred to avoid the hindrance from the broadened major peak due to the large amount of injection. In Fig. 4, the electropherogram of ractopamine separation at 10 mM S-α-CD is shown.

Separation Voltage

Another factor that may affect the separation is the separation voltage. The plots of effective mobility and R_s value vs. separation voltage are shown in Fig. 5. In this case, the mobility was almost constant in the voltage range from 10 to 30 kV. The resolution is proportional to the square root of the separation voltage.[23] Theoretically, the resolution should increase as the separation voltage increases. However, as can be seen in Fig. 5, the resolution remained constant up to 20 kV, then decreased at higher voltages. This is due to the Joule heating generated at higher voltages, which causes

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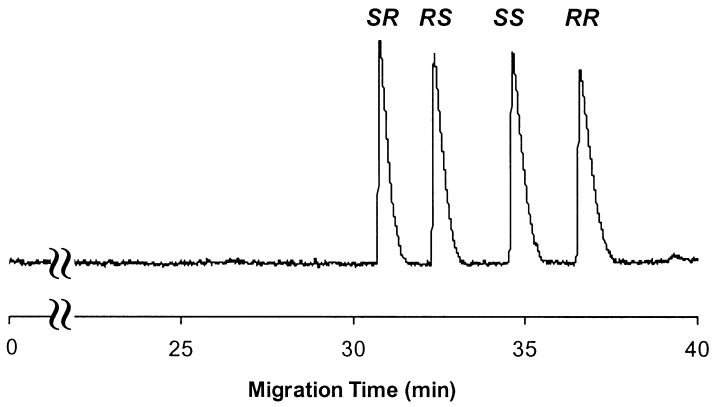

Figure 4. Electropherogram of ractopamine stereoisomers. Running buffer: 10 mM S-α-CD in 20 mM phosphate (pH 6.0), separation voltage: +20 kV, capillary temperature: 20°C.

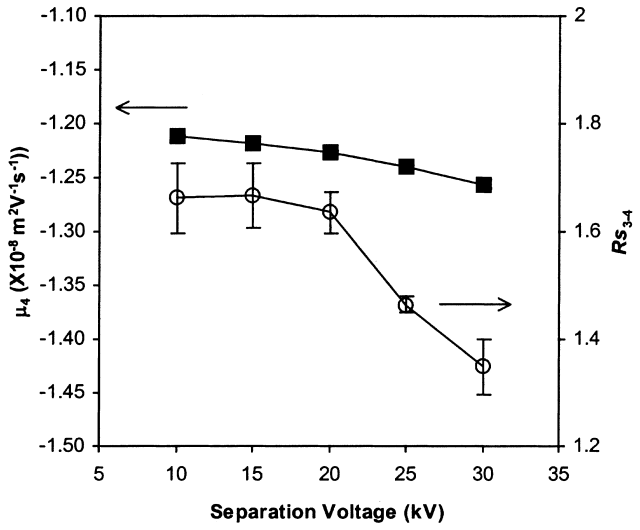

Figure 5. Effect of separation voltage on separation of ractopamine stereoisomers. *Key:* ■, relative mobility of desired peak, *RR*; ○, R_s between third and fourth eluted peaks, *SS* and *RR*. Running buffer: 4 mM S-α-CD in 20 mM phosphate (pH 6.0), capillary temperature: 20°C.

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the band broadening. For this reason, it is necessary to take care not to exceed a reasonable voltage limit. For the quantitative study, +20 kV was chosen since it showed acceptable resolution and analysis time. It should be noted that although generally the reverse polarity is used for the separation with negatively charged CDs, it did not provide sufficient separation of four isomers in the entire concentration range of S-α-CD (0 ~ 10 mM). At pH 6.0, EOF is high enough to elute all the isomers under the normal polarity.

Method Validation

In order to ensure accurate results, the method has to be evaluated by both the peak area ratio and the standard curve approaches.

Peak Area Ratio Approach

Accuracy

The less bioactive stereoisomers (*SR*, *RS*, and *SS*) were determined by the comparison of the peak area of the less bioactive isomer to the total peak area of four isomers using three solutions with different bioactive/less bioactive isomer ratios. The sample solutions were prepared by mixing four of each isomer with various ratios: (1) $RR:SR:RS:SS = 88:4:4:4$; (2) $RR:SR:RS:SS = 90:5:2:3$; and (3) $RR:SR:RS:SS = 86:3:5:6$. The total amount of the isomers in each sample solution was 0.05 mg/mL. The results were summarized in Table 2 ($n = 5$). The ratios of the less bioactive as well as bioactive components were able to be determined with high accuracy for each sample solution. The average recovery of *RR* was 99.6 ± 0.6%.

Precision

The reproducibility of the separation is one of the concerns in the validation of an assay. The data of the reproducibility in the peak area ratios from run to run within a day and from day to day are given in Table 3. These results clearly indicate the good reproducibility with RSD of ≤ 2%.

Linearity

The plots of the peak area ratio [$A_{\text{less bioactive isomer}}/(A_{\text{less bioactive isomer}} + A_{\text{total}})$] vs. the concentration ratio ([less bioactive isomer]/([less bioactive isomer] + [*RR*-isomer])) showed good linearity in concentration ratio range from 0.01 to 0.5 ($r^2 = 0.9998$, 0.9995, 0.9998 for *SR*, *RS*, *SS*, respectively).

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Table 2. Peak area ratio of the mixture of ractopamine four stereoisomers ($n = 5$).

	Peak area %	*RR*	*SR*	*RS*	*SS*
Solution 1	Actual %	88.0	4.0	4.0	4.0
	Experimental %	87.5 ± 0.4	4.1 ± 0.1	4.3 ± 0.3	4.3 ± 0.2
	RSD (%)	0.43	4.1	2.7	6.1
Solution 2	Actual %	90.0	5.0	2.0	3.0
	Experimental %	89.9 ± 0.9	4.9 ± 0.5	2.2 ± 0.2	3.0 ± 0.3
	RSD (%)	0.95	9.0	10.7	9.1
Solution 3	Actual %	86.0	3.0	5.0	6.0
	Experimental %	85.6 ± 0.4	3.1 ± 0.2	5.0 ± 0.4	6.2 ± 0.1
	RSD (%)	0.44	5.9	7.5	1.3

Note: Running buffer: 5 mM S-α-CD in 20 mM phosphate (pH 6.0); separation voltage: +20 kV; capillary temperature: 20°C.

External Standard Method

Accuracy

The absolute amount of the *RR*-ractopamine in each sample solution was also determined by the comparison of the *RR* peak area to a standard curve prepared from a reference standard of *RR*-ractopamine. Using this standard curve, the amount of *RR*-ractopamine in each of three sample solutions was calculated and the results are given in Table 4. The results showed close values to the actual amounts of *RR*-ractopamine in the solutions. The average recovery of *RR* was $97.6 \pm 6.1\%$.

Precision

In Table 5, the run-to-run and day-to-day reproducibility by the external standard method is summarized. The RSD values for both run-to-run and day-to-day were $\leq 4\%$, which were larger than those obtained by the peak area ratio method.

Linearity

The calibration plots for all of the ractopamine isomers were constructed by plotting peak area vs. concentration of each isomer in the range of 0.5 μg/mL to 1 mg/mL. Each isomer gave good linearity within this range ($r^2 = 0.9998$, 0.9998, 0.9995, and 0.9997 for *RR*, *SR*, *RS*, and *SS*-isomer, respectively).

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Table 3. Reproducibility in the relative peak area of each ractopamine stereoisomer: (1) run-to-run within a day, and (2) day-to-day.

	(1) Run-to-run within a day					(2) Day-to-day				
	Relative peak area (%)					Relative peak area (%)				
	Run 1	Run 2	Run 3	Average	RSD (%)	Day 1	Day 2	Day 3	Average	RSD (%)
RS	24.8	24.5	24.7	24.7	0.6	24.7	24.9	25.1	24.9	0.8
SR	24.7	24.4	24.9	24.7	1.0	24.7	24.3	24.3	24.3	0.9
SS	25.1	26.0	25.2	25.4	1.9	25.4	25.5	25.6	25.5	0.4
RR	25.4	25.1	25.2	25.2	0.6	25.2	25.3	25.3	25.3	0.2

Note: Running buffer: 4 mM S-α-CD in 20 mM phosphate (pH 6.0); Separation voltage: +20 kV; Capillary temperature: 20°C; Sample concentration: 0.2 mg/mL.

Table 4. Determination of the amount of *RR*-ractopamine in the mixture of four stereoisomers using a standard curve prepared from a reference standard of *RR*-ractopamine ($n = 5$).

	Solution 1	Solution 2	Solution 3
Actual (mg/mL)	0.044	0.045	0.043
Experimental (mg/mL)	0.042 ± 0.002	0.047 ± 0.003	0.040 ± 0.003
RSD (%)	5.9	5.6	7.1

Note: The experimental conditions are the same as described in Table 2.

Trace Levels of the Less Bioactive Isomers

For determination of the trace levels of impurities ($\leq 1\%$), high concentration of the sample solution is usually required due to the limited sensitivity to the minor impurities, which may cause severe peak broadening. Sufficiently large resolution is necessary to avoid co-elution of impurities with the major component. For this reason, the concentration of S-α-CD in the running buffer was increased to 10 mM in this study. Figure 6(a), (b) are the examples of the electropherograms for determination of the less bioactive isomers of ractopamine. The limit of quantitation (LOQ) of each isomer was 0.2, 0.2, and 0.5% (wt.%) for *SR*, *RS*, and *SS*, respectively. LOQ of *SS*-ractopamine was poorer than the other two isomers due to the adjacency of the *SS*-isomer peak to the major *RR*-isomer peak, which caused overlapping with *RR*-isomer peak. The peaks of *SR*-and *RS*-isomer were separated well enough from the *RR*-peak to enable the LOQ of 0.2%.

Determination of Ractopamine Stereoisomers in Feed Premix for Swine

Ractopamine stereoisomers in the feed premix for swine (contains 2% of ractopamine with nearly equal amounts of each isomer) were extracted and determined using the optimized method. In Table 6, the peak area ratios of the isomers obtained from ractopamine reference standard and from the feed premix are compared. No significant difference in the ratios was observed between the feed premix and the reference standard. Isomerization does not seem to occur during the processes of the feed premix preparation, as well as the storage of the feed premix. The recovery of *RR* isomer was $92.6 \pm 4.6\%$.

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Table 5. (1) Run-to-run and (2) day-to-day reproducibility of each ractopamine stereoisomer by the external standard method.

	(1) Run-to-run					(2) Day-to-day				
	Amount, μg/mL					Amount, mg/mL				
	Run 1	Run 2	Run 3	Average	RSD (%)	Day 1	Day 2	Day 3	Average	RSD (%)
RS	47.5	50.5	49.0	49.0	3.1	49.2	49.0	49.2	49.1	0.2
SR	46.6	48.6	50.5	48.6	4.0	47.8	50.5	48.6	49.0	2.8
SS	50.7	50.6	51.4	50.9	0.9	52.2	51.4	50.0	51.2	2.2
RR	50.5	49.4	51.8	50.6	2.4	52.2	51.8	49.0	51.0	3.4

Note: Experimental conditions are the same as described in Table 3.

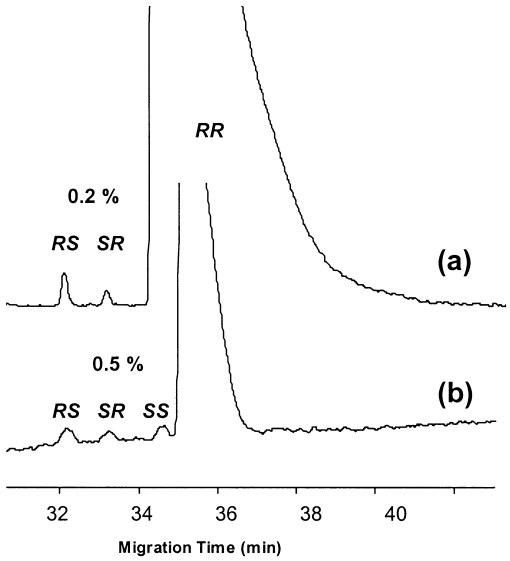

Figure 6. Determination of trace amounts of isomeric impurities. Sample solution: (a) 1.5 mg/mL of *RR*-ractopamine + 3 μg/mL of *SR*-, *RS*-, and *SS*-ractopamine (0.2% each) in water, (b) 0.5 mg/mL of *RR*-ractopamine + 2.5 μg/mL of *SR*-, *RS*-, and *SS*-ractopamine (0.5% each) in water. Concentration of S-α-CD: 10 mM, pH: 6.0, separation voltage: +20 kV, separation voltage: +20 kV, capillary temperature: 20°C.

CONCLUSIONS

Four stereoisomers of ractopamine were successfully separated by CE using either HDAS-β-CD or S-α-CD as a chiral selector. The buffer pH, concentration of the selector, and separation voltage showed significant effects on separation and these parameters were optimized using S-α-CD to obtain better separation. The method showed good reproducibility and usefulness for

Table 6. Comparison of the peak area ratios of ractopamine stereoisomers in the reference standard and in the premix feed.

	RR, %	*SR*, %	*RS*, %	*SS*, %
Reference STD	25.6	24.6	24.2	25.6
Premix feed	25.9	25.0	23.6	25.5

Note: The experimental conditions are the same as described in Table 3.

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the quantitative analysis, especially determination of the trace amounts of the isomers. The analysis of ractopamine isomers extracted from the feed premix for swine was possible using the optimized method.

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