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Separation and determination of norepinephrine, epinephrine and isoprinaline enantiomers by capillary electrophoresis in pharmaceutical formulation and human serum

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Abstract

A capillary electrophoresis method with ultraviolet (UV) detection was developed and optimized for the enantiomer separation of norepinephrine (NE), epinephrine (EP) and isoprenaline (IP) using dual cyclodextrins (CDs) of 2-hydroxypropyl-β-CD (HP-β-CD) and heptakis (2,6-di-o-methyl)-β-CD (DM-β-CD) as chiral selectors. Optimal separation was obtained using a running buffer of 50 mM phosphate containing 30 mM HP-β-CD and 5 mM DM-β-CD at pH 2.90 and a field strength of 20 kV in 45 cm × 75 μm (40 cm effective length) uncoated capillary. The UV absorbance detection was set at 205 nm. A 0.1% (w/w) polyethylene glycol or 0.1% (v/v) acetonitrile was used to enhance the detection sensitivity. There was a wide and excellent linear calibration graph for each enantiomer in the range 1.0×10^{-3} to 1.0×10^{-6} M and the detection limit (S/N = 3) was found from 8.5×10^{-7} to 9.5×10^{-7} M. The method has been applied for the determination of isoprenaline in isoprenaline hydrochloride aerosol and to the analysis of serum samples. The recoveries of NE and EP in serum and IP in drug were ranged from 90 to 110%. The relative standard deviations of all the analyte peaks were less than 2.8% for migration time and less than 4.8% for peak area.

Keywords: Capillary electrophoresis; Cyclodextrin; Enantiomer separation; Norepinephrine (NE); Epinephrine (EP); Isoprinaline (IP)

1. Introduction

Isoprenaline is symphatomimetic drugs with potent β_2-adrenoceptor widely used in the treatment of respiratory diseases [1]. It is also used illegally as growth promoters in the liver stock industry [2]. Its residue can be toxic to humans. Epinephrine (EP) and norepinephrine (NE), component of neural transmission media, have important effect on the transmission of nerve impulses and exhibit vasoconstriction and blood pressure elevation. Many diseases such as Parkinson's are related to the concentration of EP and NE in blood as well as urine. Pharmaceutically, they are widely used for treatment of neutral disorder [3]. These drugs are usually administrated as the racemate, which exhibit very different pharmacological effects. For example, the activity of L-EP is ten times stronger than its isomer D-EP [4]. Therefore, L-forms are usually used in the local anesthetic or ophthalmic solution. In order to monitor the effect of drugs given, to control the enantiomeric purity of the drugs administered and to study the drug pharmacokinetic and pharmacodynamic properties, it is desired and important to develop the analytical method that can effectively separate and determine these enantiomers.

A number of analytical methods have been reported to determine EP, NE and isoprenaline (IP) enantiomers [5–19]. Among them, high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) are the most widely used separation techniques. For example, the enantiomer separation of both NP and EP by HPLC was reported after derivatization [5,6] or directly separation [7–9]. However, these HPLC methods suffer a few drawbacks, such as by-products derived disturb the detection of the derivatives in the chromatogram, lack of general applicability, high consumption of samples and less robust.

CE is a powerful enantiomer separation and quantitation technique that often provides higher separation efficiency, shorter

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analysis time, lower operation cost, more robust and wider applicability. CE with the direct method using β-cyclodextrin (β-CD) or derivatized-CD as chiral selectors was reported to be effective for enantiomer separations of NE, EP and IP. Quang and Khaledi [10] reported the use of β-CD and tetraalkylammonium for the separation of NE, EP and IP enantiomers. However, only partial separation was obtained for NE and EP. Gahm and Stalcup [11] and Yang et al. [12] investigated the enantiomer separation of NE, EP and IP using sulphated β-CD as chiral selector. The resolutions of NE and EP were insufficient and the peak of each enantiomer tailed. Male and Luong [13] demonstrated DM-β-CD as chiral selector to separate NE, EP and IP, but the separation of NE and EP could not be improved. Dong and Sun [14] and Liu and Nussbaum [15], respectively, reported baseline separation of IP by using HP-β-CD, or DM-β-CD, CM-β-CD as chiral selector. Garcia-Ruiz and Marina [16] and Cucinotta et al. [17] studied the enantiomer separation of NE and EP by using permethylated-β-CD or 6-O-succinil-β-CD as chiral selector, however, the tested of NE and EP enantiomers could not be completely resolved. Recently, Schwarz and Hauser [18] demonstrated a combination of CM-β-CD and 18-crown-6 as chiral selector to separate the enantiomers of NE and EP, but the baseline separation of the two forms of NE was not successful. Therefore, in spite of intensive investigates in the field of enantiomer separation of NE, EP and IP, the resolution achieved for NE or EP was insufficient to permit practical use. To the best of our knowledge, until now no application to biofluids, enantiopurity testing or commercial formulations have been reported. To suit practical use, a further improvement of the resolution is required.

In this study, we attempted to improve the separation of enantiomers of NE and EP by using HP-β-CD or HP-β-CD combination of other selectors. In additional, organic modifier has been tried and found effective in order to increase the method detection sensitivity. The developed assay method was also validated for sensitivity, precision, linearity, recovery, and reproducibility. It has been successfully applied to the analysis pharmaceutical formulation and human serum samples.

2. Experimental

2.1. Reagents

(±)-Norepinephrine L-bitartrate hydrate (99%) (NE), (R)-(−)-norepinephrine hydrochloride bitartrate salt, (±)-epinephrine hydrochloride (EP) and (±)-isoprenaline hydrochloride (IP) (structural formulate was shown in Fig. 1) were purchased from Sigma Aldrich (St. Louis, MO, USA). Isoprenaline hydrochloride aerosol was obtained from Penglai Nuokang Pharmaceutical Factory (Shandong, China). β-Cyclodextrin (β-CD) was obtained from Fluka (Buchs, Switzerland). Methyl-β-cyclodextrin (Me- β-CD) and dimethyl-β-cyclodextrin (DM-β-CD) were from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Hydroxypropyl-β-cyclodextrin (HP-β-CD, >97%) was purchased from Acros Organics. Polyethylene glycol was obtained from Shanghai Chemical Reagent Co. (Shanghai, China). Tris (hydroxymethyl) aminomethane (Tris), tetramethylammoniumhydroxide solution (TMAH), phosphoric acid, acetone (≥99.5%) and triethanolamine (TEA) were purchased from Beijing Chemical Factory (Beijing, China). Acetonitrile and ethylene glycol were from Tianjing Chemical Factory (Tianjing, China). All other reagents used were of analytical grade purity. Water for preparation of sample and buffer solution was deionized by a Milli-Q purification system with a 0.2-μm fiber filter (Barnstead, CA, USA).

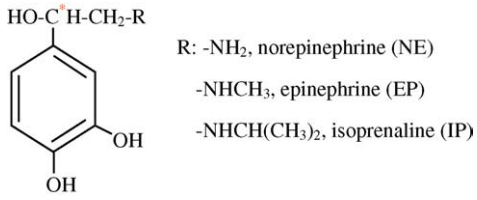

Fig. 1. Chemical structures of norepinephrine, epinephrine and isoprinaline.

2.2. Apparatus and analytical procedure

All experiments were performed on a Beckman P/ACE MDQ capillary electrophoresis system (Beckman, Fullercon, CA, USA) equipped with a photodiode array detection system. Sample detection was performed at 205 nm. The electropherograms were recorded and integrated by an IBM personal computer with 32-karat software version 4.0 (Beckman). An uncoated fused-silica capillary (Yongnian Optic Fiber Factory, Heibei, China) of 75 μm I.D. and 40 cm effective length was used for separation. The capillary temperature was maintained at 25 °C by the cooling system of the CE instrument. The new capillary was flushed successively for 30 min with 1 M NaOH, 10 min with 1 M HCl, 10 min with water to activate and clean the silica wall and then equilibrated with the operating buffer for 10 min. After every running the capillary was preconditioned with 1 M NaOH for 2 min, water for 2 min, the running buffer for 2 min. At the beginning of each day and whenever the buffer solution was changed, the capillary was rinsed with 1 M NaOH for 5 min, water for 2 min, and the running buffer for 2 min. Samples were injected with pressure at 0.8 psi for 10 s and separated at 20 kV (1 psi = 6894.76 Pa). Standard solutions of EP and IP were prepared by dissolving each of them in water to give a concentration of 10^{-3} M and 7×10^{-4} M, respectively. Finally, dilutions of this solution in water were made in order to obtain the required concentration. The background electrolyte (BGE) was prepared by mixing a 50 mM phosphoric acid, 30 mM HP-β-CD, 5 mM DM-β-CD, 0.1% (w/w) polyethylene glycol in water and pH adjusted to 2.90 with pH meter (IQ Scientific Instruments, Shanghai, China) by adding TEA or TMAH, 0.5 M Tris solution.

2.3. Sample preparation

The determination of the IP in the isoprenaline hydrochloride aerosol required a dilution of the aerosol in a final volume of 1.0 mL with water. Different sample solutions were prepared by diluting different amounts of the aerosol

in water in order to obtain the desired concentration. The resulting solution was directly injected into the electrophoretic system.

Human serum samples were obtained from the Hospital of Henan Normal University (Henan, China). A 1.00 mL serum sample was extracted with 2 mL acetonitrile mixture by vortex mixing for 2 min followed by centrifugation at 3500 rpm for 10 min. The organic layer was recovered and dried to 1 mL under a gentle nitrogen stream, and then injected for electrophoresis.

2.4. Data analysis

The corrected peak areas were calculated using Beckman P/ACE station software system version 4.0. The enantiomeric resolution (R_s) was calculated according to the equation

$$R_s = \frac{2(t_2 - t_1)}{w_2 + w_1} \quad (1)$$

where t_1 and t_2 are the migration time and w_1, w_2 are the corresponding widths at the base of the peaks.

3. Results and discussion

3.1. Preliminary experiments

In order to develop a robust method with low cost and suitable practical use, three relatively cheap CDs: β-CD, Me-β-CD and HP-β-CD were selected for preliminary experiments. Experiments were performed with the BGE composed of 75 mM H_3PO_4 and 15 mM different CD at pH 2.5 titrated with TMAH. No enantiomer separation was observed for NE and EP with β-CD or Me-β-CD. Only partially enantiomer separation of IP was achieved using β-CD or Me-β-CD. Surprisingly, when HP-β-CD was used, the enantiomer separation of both NE and EP was observed, and excellent baseline enantiomeric separation of IP was obtained. It can be deduced from the peak order that IP binds most strongly to HP-β-CD followed by EP and NE, which is consistent with the conclusion that stronger binding results in better resolution. Therefore, HP-β-CD was used for the method optimization.

3.2. Effect of pH, buffer type and concentration

As shown in Fig. 2, the resolutions of all three pairs of enantiomer decreased with increasing pH values. The best resolutions were obtained at pH 2.80–3.10. Therefore, pH 2.90 was selected as optimum. At pH 2.90, the effect of the four different buffers, H_3PO_4–Tris, H_3PO_4–TMAH, H_3PO_4–TEA and H_3PO_4–NaOH on the enantiomer separation using HP-β-CD as selector were compared. The results showed that the addition of Tris or TEA to BGE can markedly reduce the tailing of the peak and significantly improve the resolution. The buffer containing Tris gives higher resolution and shorter migration times than that of TEA. However, it provides lower sensitivity than TEA, and when H_3PO_4–Tris buffer solution was used to analyze biological

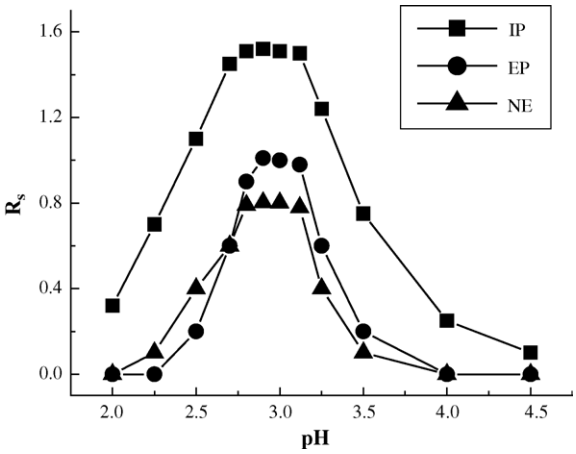

Fig. 2. Effect of pH on the resolution. Conditions: uncoated fused-silica capillary, 47 cm (length to detector 40 cm) × 75 μm I.D.; buffer: 30 mM H_3PO_4 containing 15 mM HP-β-CD at the desired pH titrated with tetramethylammoniumhydroxide solution (TMHA); separation voltage: 15 kV; detection 205 nm; temperature 25 °C; hydrodynamic injection 5 psi for 8 s. IP, isoprinaline; EP, epinephrine, NE, norepinephrine.

sample, the matrix substances interfered seriously the determination of norepinephrine. Thus, H_3PO_4–TEA was selected as the components of running buffer.

A concentration range of 25, 50, 75 and 100 mM H_3PO_4–TEA was tested while the HP-β-CD concentration was maintained at 15 mM with pH 2.90. The sensitivity of each analyte decreased as BGE concentration increased. The resolution of NE was not improved when the concentrations of BGE was above 50 mM. Taking into consideration of sensitivity and resolution simultaneously, 50 mM was selected.

3.3. Effect of CD concentration on enantiomer separation

According to the equation for the apparent mobility of enantiomers [19],

$$\Delta\mu = \frac{[\mathrm{CD}](\mu_f - \mu_c)(K_1 - K_2)}{(1 + [\mathrm{CD}]\,(K_1 + K_2) + K_1K_2)[\mathrm{CD}]^2)} \quad (2)$$

where [CD] is the CD concentration of the chiral selector; μ_f and μ_c are the electrophoretic mobility of the free analyte and complex solutes, respectively; K_1 and K_2 are the binding constants of the individual enantiomers with the chiral selector.

The mobility difference between the enantiomers is dependent on the binding constants and the CD concentration. Fig. 3 shows the effect of concentration of HP-β-CD on enantiomer separation. It can be seen that the apparent mobility difference increased as the concentration of HP-β-CD increased, reaching a maximum value for NE, EP and IP at 30 mM. Above 30 mM, the enantiomeric separation of NE or EP could not be improved and the migration time increased. Many papers [10,13,20] reported that using dual CD system could obtain higher enantiomeric resolutions. Therefore, in our work, 5 mM of β-CD, Me-β-CD or DM-β-CD was added to the running buffer containing 30 mM HP-β-CD, respectively. The results demonstrated that no significantly improvement of resolution for NE or EP was observed

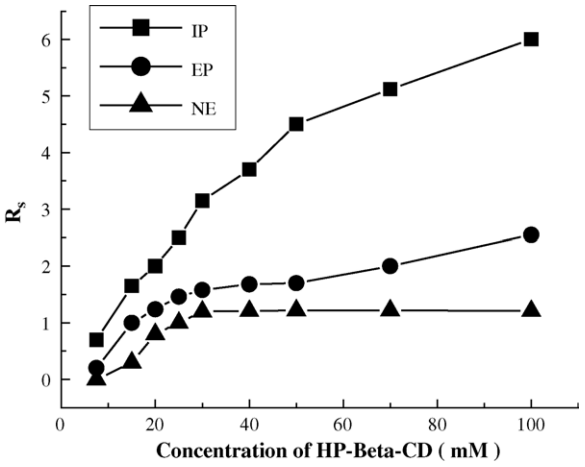

Fig. 3. Effect of concentration of HP-β-CD on chiral separation. Buffer: 50 mM H_3PO_4–TEA containing different concentration of HP-β-CD at pH 2.90; other conditions were the same as Fig. 2. IP, isoprinaline; EP, epinephrine, NE, norepinephrine.

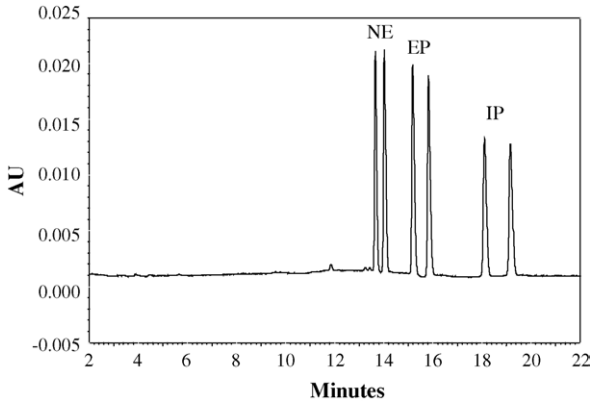

Fig. 4. Typical electropherogram of 5.00×10^{-5} M NE and EP, and 3.0×10^{-5} M IP standard solution. Uncoated fused-silica capillary, 47 cm (length to detector 40 cm) × 75 μm I.D.; buffer: 50 mM H_3PO_4–TEA containing 30 mM HP-β-CD + 5 mM DM-β-CD and 0.1% polyethylene glycol at pH 2.90; separation voltage: 20 kV; detection 205 nm; temperature 25 °C; hydrodynamic injection 0.8 psi for 10 s.

with the addition of β-CD or Me-β-CD. While the addition of DM-β-CD to the running buffer containing HP-β-CD resulted in a greater resolution for NE and did not strongly affect the analysis time. These results clearly indicated that the hydrophobicity of DM-β-CD promotes inclusion of the aromatic ring and enhances the enantioselectivity of NE. Using the mixture of 30 mM HP-β-CD and 5 mM DM-β-CD, a best separation of three enantiomer pairs of NE, EP and IP could be achieved simultaneously.

3.4. Influence of organic modifier

Five organic solvents, methanol, acetone, acetonitrile, ethylene glycol and polyethylene glycol were investigated in a 0.01–5% (v/v) amount range. The low concentration of these organic solvents improved the resolution, but when the concentration of methanol, acetone or ethylene glycol reached 0.1%, and acetonitrile or polyethylene glycol reached 0.2%, it was unable to get the baseline separation for 1 mM NE. Among these solvents, a 0.1% (v/v) polyethylene glycol improved not only the resolution but also detection sensitivity. Considering both resolution and detection sensitivity, a 0.1% polyethylene glycol was used in this work.

3.5. Effect of injection time

Another way to enhance the detection sensitivity is to apply high sample volume to the capillary. The injection time between 3 and 20 s was studied using hydrodynamical injection at 0.8 psi. It was found that the peak height and area were increased linearly over the injection time from 3 to 10 s. Above 10 s, the peak became broader resulting in a poor separation of NE enantiomer. Therefore, the injection time of 10 s was chosen.

3.6. Validation

Under the above optimum conditions, a typical electropherogram of NE, EP and IP was shown in Fig. 4. Three pairs of enantiomers were separated well. The validation of this method was performed for the quantification of IP in a pharmaceutical aerosol and to the analysis of serum samples. The quantification analysis was performed using the external standard method for calibration under optimized conditions. The linearity of the method was assessed using seven standard solutions in the range of 1.0–1000 μM. As shown in Table 1, calibration curves for NE, EP and IP enantiomers were found to be linear. The correlation coefficients were ranged from 0.9988 to 0.9999.

Table 1
Repeatability and linearity data

Enantiomer	*a*	*b*	*r*	Linear range (μM)	RSD (%) (*n* = 5)		LOD (μM)
					RMT	RPA	
R-norepinephrine	−137.79	2.38×10^9	0.9988	1.0–1000	0.90	1.92	0.85
S-norepinephrine	−146.85	2.61×10^9	0.9998	1.0–1000	1.30	2.15	0.85
R-epinephrine	−434.39	2.63×10^9	0.9994	1.0–1000	0.95	1.98	0.95
S-epinephrine	−893.21	2.77×10^9	0.9988	1.0–1000	1.12	1.72	0.95
R-isoprenaline	−204.83	2.18×10^9	0.9992	1.0–1000	1.50	2.37	0.90
S-isoprenaline	−260.05	2.29×10^9	0.9999	1.0–1000	0.85	2.48	0.90

Calibration curves are expressed as regression lines ($y = a + bx$), where y is integrated peak area and x is concentration of enantiomenr (μM). a, b and r are intercept, slope and relative coefficient, respectively. RSD is relative standard deviation and was determined for five consecutive injections. RTM, relative migration time; RPA, relative peak area; LOD is limit of detection at 3:1 signal-to-noise ratio.

The limit of detection was defined as signal-to-noise ratio equal to three times the standard deviation. It ranged from 0.85 to 0.95 μM. Compared with the reported sensitivities of NE and EP of 0.8–0.95 μM by micellar electrokinetic capillary chromatography (MEKC) [21] and 0.5–4 μM [22] after solid-phase extraction (SPE) purification by UV-CE without derivatization. The sensitivity of this method is high enough for our selected samples.

The precision of the method was studied by repeatability and reproducibility. The repeatability in peak area and migration time of the enantiomers (expressed as relative standard deviation, RSD) was determined for five consecutive injections of a standard solution. RSD values of migration times were less than 2.0%. The RSD values of the peak area were below 3.0%. To assess the reproducibility, five determination of each enantiomer compound was made on five different days. In this case, the RSD for migration times was less than 2.8% and less than 4.8% for peak area (data not shown).

The accuracy of the method was evaluated by recovery studies. The serum samples spiked with NE and EP at the different concentrations were injected by triplicate. The results were summarized in Table 2. The recoveries ranged from 90 to 110% with satisfactory results. Good precision and accuracy indicated that the method was reliable.

3.7. Analytical application for human serum and pharmaceutical formulation

The present method was applied to the analysis of serum samples. Fig. 5a shows the electropherograms of serum samples extraction. Peak identification was made with the method of standard solution addition and shown in Fig. 5b. It can be seen that the serum samples matrix did not interfere with the determination of the analytes of interest and *R*-NE could be determined sensitively, whereas *S*-NE, *R*-EP and *S*-EP were not detected. The average concentration of *R*-NE found in serum samples extraction based on three replicate injections was 1.0 μM. The concentration found in serum samples extraction with the present method was approximately 20% higher than the ones obtained in urine with CE–MS method [23].

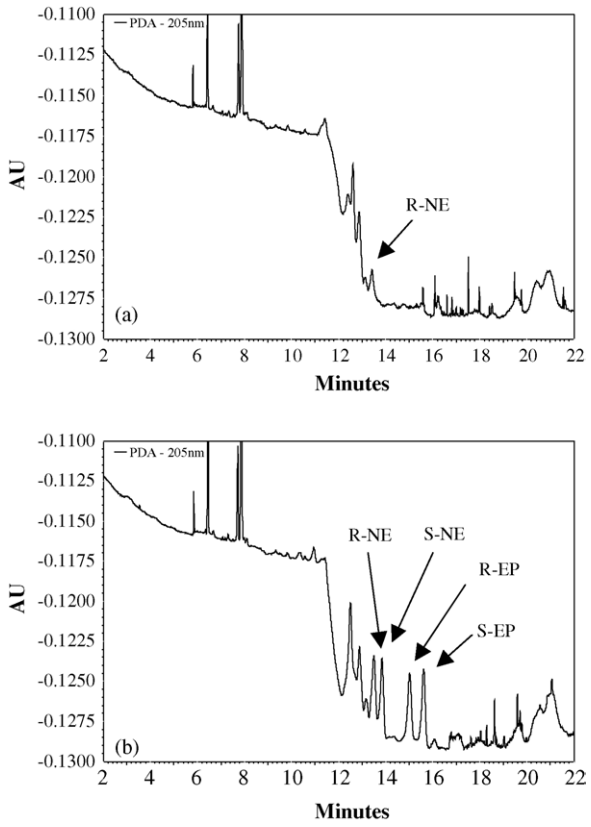

Fig. 5. Analysis of extracted serum sample: (a) blank serum profile; (b) serum sample spiked with 1.00×10^{-5} M of NE and EP standard solution; Experimental conditions as in Fig. 4.

Table 2
Accuracy estimated as recovery (%) of urine or serum spiked with NE, E and IP at three different concentrations

Analyte	Serum samples		
	Added (M)	Found[a] (M)	Recovery (%)
R-NE	–	1.0×10^{-6}	–
	1.0×10^{-5}	9.5×10^{-6}	95
	1.0×10^{-4}	9.3×10^{-5}	93
	5.0×10^{-4}	4.5×10^{-4}	90
S-NE	–	ND[b]	–
	1.0×10^{-5}	9.2×10^{-6}	92
	1.0×10^{-4}	9.7×10^{-5}	97
	5.0×10^{-4}	4.7×10^{-4}	94
R-EP	–	ND[b]	–
	1.0×10^{-5}	9.4×10^{-6}	94
	1.0×10^{-4}	1.1×10^{-4}	110
	5.0×10^{-4}	4.9×10^{-4}	98
S-EP	–	ND[b]	–
	1.0×10^{-5}	9.6×10^{-6}	96
	1.0×10^{-4}	1.0×10^{-4}	100
	5.0×10^{-4}	4.8×10^{-4}	96

[a] Average of three measurements.
[b] Not detectable.

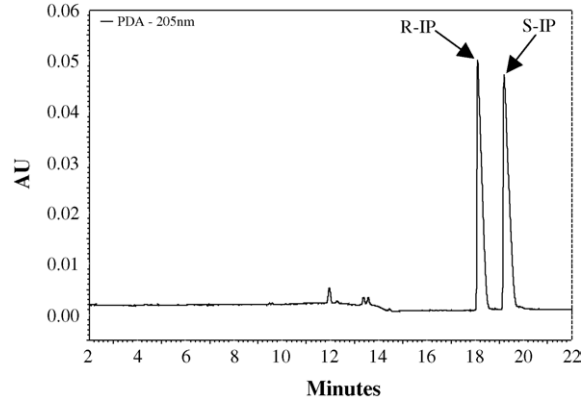

Fig. 6. Typical electropherograms of a 0.175 mg/mL isoprenaline aerosol sample. Conditions were as same as in Fig. 4. Peaks: (1) *R*-IP, (2) *S*-IP.

The proposed method was also used to determine IP in the pharmaceutical product (an aerosol solution with 0.175 mg/press of IP). Fig. 6 shows a typical electropherograms of one press aerosol sample in 1.00 mL water. The peaks were identified by standard addition under the exactly same conditions. The first migration peak was identified as *R*-forms and the second migration peak was identified as *S*-forms. The average values of three determinations obtained by the calibration and the standard addition method were 0.170 ± 0.005 mg/mL and 0.172 ± 0.005 mg/mL, respectively. They were excellently agreement with the labeled value (0.175 mg/mL).

4. Conclusions

A capillary zone electrophoresis method using HP-β-CD and DM-β-CD as chiral selector has been developed for the simultaneous separation and quantitation of norepinephrine, epinephrine and isoprenaline enantiomers. The study has shown that the use of DM-β-CD could improve the chiral separations. Polyethylene glycol was favorable to improve the efficiency of the enantiomer separation and the detection sensitivity. The validation of this method for the quantitation of isoprenaline aerosol and to the analysis of serum samples spiked norepinephrine and epinephrine was performed. Compared with the previous methods [10–18], the merit of this work was used to analyze enantiomers of norepinephrine, epinephrine or isoprinaline in pharmaceutical formulation and human serum. The results indicated the proposed method represents good selectivity, precision and accuracy. It is a simple, inexpensive and does not require any complicated sample preparation of method, which can be applied successfully to the identification and quantification of *R*-NE in serum without the interference of matrix. It also promises to be applicable to the quality or purity control of pharmaceutical products.

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