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Short communication

Determination of β_2-agonists by ion chromatography with direct conductivity detection

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Abstract

A simple method for the simultaneous detection of four β_2-agonists (salbutamol, fenoterol, clorprenaline, and clenbuterol) using ion chromatography (IC) with direct conductivity detection (CD) based on their ionization in acidic medium without chemical suppression is presented. The mixture of 1.8 mM HNO_3 and 2% (v/v) acetonitrile was used as eluent. The method could be applied to the determination of the β_2-agonists in pharmaceutical preparations. The recovery of salbutamol and clenbuterol in tablets was more than 97% ($n = 3$) and the relative standard deviation ($n = 11$) less than 2.8%. With the proposed method, salbutamol could also be successfully detected in human plasma. In a single chromatographic run, the four β_2-agonists can be separated and determined in less than 8 min. The linear ranges were of 7.0–1.4 $\times 10^3$ ng/ml for salbutamol, 34–7.8 $\times 10^3$ ng/ml for fenoterol, 8.0–1.6 $\times 10^3$ ng/ml for clorprenaline, and 25–7.5 $\times 10^3$ ng/ml for clenbuterol. The detection limits were 2.0 ng/ml for salbutamol, 10 ng/ml for fenoterol, 3.0 ng/ml for clorprenaline, and 10 ng/ml for clenbuterol.

Keywords: β_2-Agonists; Salbutamol; Fenoterol; Clorprenaline; Clenbuterol; Ion chromatography; Conductivity detection

1. Introduction

Ion chromatography (IC) has developed into the method of choice for the simultaneous determination of mixtures of inorganic anions or cations. The technique has also often been extended to the determination of low molecular mass organic ionic species, such as C_1–C_5 carboxylic acids, sulfonic acids, amines, etc. The application of IC to larger organic ions is much less common. However, theoretically, this technique could also be used for the separation of organic compounds with relatively high molecular weights. There are broad opportunities to use IC to detect larger organic ions [1].

β_2-Agonists such as salbutamol, fenoterol, clorprenaline and clenbuterol can effectively prevent and reverse bronchoconstriction; they are the most powerful bronchodilators currently available and can be used to treat pulmonary diseases of humans and animals [2–5]. Furthermore, β_2-agonist compounds are used in animal rearing as growth promoters [6]. However, the danger from the residues resulting from the abuse of these agents has been underscored by several human poisoning incidences where the consumption of animal food products containing clenbuterol residues was implicated. It is, therefore, necessary to develop rapid and sensitive methods for the separation and detection of β_2-agonists.

Various analytical methods for the determination of β_2-agonists have been described [7–16]. Gas chromatography–mass spectrometry (GC–MS) [7–9] is a common method to detect these bronchodilators. However, a derivatisation step is required prior to injection, which is complicated and time-consuming [13]. Furthermore, the derivatisation procedures proposed for β_2-agonist are not entirely satisfactory

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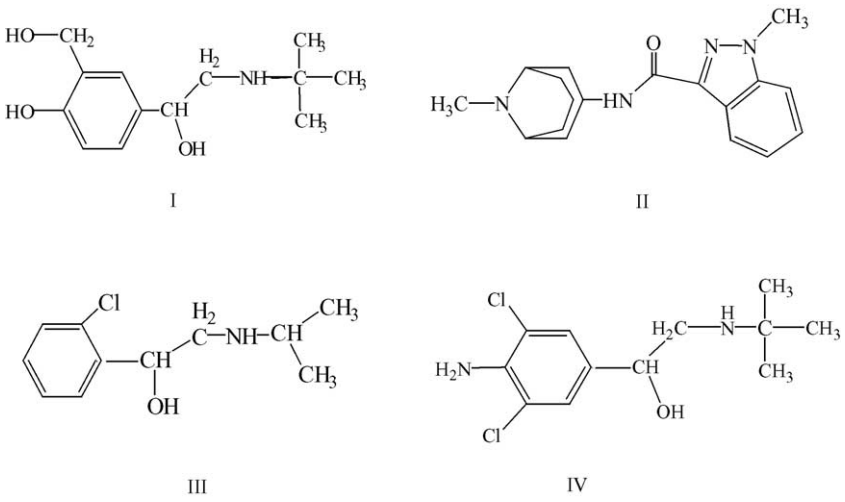

Fig. 1. Structure of four β_2-agonists: I salbutamol; II fenoterol; III clorprenaline; IV clenbuterol.

owing to the low specifity of the resulting electron impact (EI) mass spectra or to the restricted range of applicability. HPLC offers advantages over GC to detect β_2-agonists [13]. The HPLC methods reported all used columns with reserved-phase sorbents with buffered mobile phase, either with or without an ion-pair reagent [17]. UV detection is the most popular detection method applied in HPLC. However, different β_2-agonists have different UV characteristics, the β_2-agonist such as salbutamol and fenoterol do not sufficiently absorb UV light as a result of which HPLC methods with UV detection cannot offer enough sensitivity for the simultaneous detection of a group of β_2-agonists sensitively at a single wavelength UV detection [13,18]. UV detection for β_2-agonists gives good sensitivity only after a post-column derivatisation procedure [19], but it is complicated and time-consuming. Moreover, UV detection cannot offer sufficient selectivity for the determination of β_2-agonist [13] because the complicated matrix would also absorb UV light, causing interference for β_2-agonist detection. In recent years, fluorescence detection has been extensively applied as the detection method in liquid chromatography, as low detection limit can be achieved. However, this includes the need for applying a variety of sample clean-up procedures, so as to decrease background interference from the sample matrix. This usually is the slowest step of the analysis. Solid phase extraction (SPE) has become one of the most popular techniques for sample clean-up procedure in recent years. SPE offers good recoveries and can be automated; however, higher investments are needed. Liquid chromatographic–mass spectrometry (LC–MS) detection methods have been developed for the determination of β_2-agonist intensely [19,20]. However, the powerful and expensive LC–MS equipment does not belong to the facilities of average investigator.

Our preliminary [21] experiments demonstrated that organic compounds with relatively high molecular weights could be ionized in aqueous solution at relatively low pH values. The structure of the four β_2-agonists were shown in Fig. 1; it can be observed that they can ionize in aqueous solution at relatively low pH values. These analytes therefore could be separated on the stationary phase mainly based on ion exchange. They could be detected online by a conductivity detector available in the commercial ion-chromatographic instrument sensitively. To avoid interferences, β_2-agonists could be first extracted from plasma by toluene, and then reversibly extracted by using the diluted HNO_3 solution. The latter solution was then utilized as mobile phase for the ion chromatographic separation. The determination of salbutamol in plasma is important for clinical treatment. Limited data have been published on the pharmacokinetics of salbutamol. Moreover, there are no data on the extent to which inhaled salbutamol undergoes first-pass metabolism. This lack of information is most likely due to the very low plasma concentrations reached after inhalation of therapeutic doses of salbutamol, and the problems in developing an analytical method that is sensitive enough to determine these concentrations [22]. Using this method, we successfully determined salbutamol in plasma. This sample extraction procedure is time saving, cheap and may improve selectivity of analysis. Moreover, the proposed composition of the mobile phase is quite simple, only diluted acid is used for the separation and the analysis time is short (all analytes can be detected within 8 min).

2. Experimental

2.1. Materials

Salbutamol, fenoterol, clenbuterol and clorprenaline were purchased from the National Institute for the Control of Pharmaceutical and Biological Products of the People's Republic of China. Nitric acid, toluene and acetonitrile were obtained from Beijng Yili Chemical Ltd. Company. All other

chemicals used in this paper were of analytical reagent grade. Deionised water (Millipore MilliQ Water System, USA) was used throughout. The mobile phase was 1.8 mM nitric acid containing 2% (v/v) of acetonitrile. The stock standard solutions containing 250 μg/ml of the four β_2-agonists were prepared by dissolving salbutamol, fenoterol, clorprenaline and clenbuterol in the mobile phase, respectively. The stock standard solutions were stored at 4 °C for 1 week.

2.2. Equipment

A Metrohm ion chromatography apparatus (Metrohm, Switzerland) was used consisting of a 733 IC separation center, a 709 IC pump detector and a 732 IC detector. A Metrohm cation 1–2 separation column was used for the separation. All instrument control and data collection was performed by Metrohm chromatography software IC Metrodata for Windows.

2.3. Chromatographic conditions

A Metrohm cation-exchange column Metrosep cation 1–2 with a cationic pre-column was used for the separation. The mobile phase was a mixture of 1.8 mM HNO_3 and 2% (v/v) acetonitrile. Injection volume: 20 μl, flow-rate: 1.0 ml/min. β_2-Agonist peaks were identified by comparing the retention times from the sample solution with those from the standard solutions. The contents of salbutamol, fenoterol, clorprenaline and clenbuterol were quantified by comparing peak heights in the elution profile of the sample with those of known standards.

2.4. The extraction procedure of plasma

Four hundred microliters plasma was added to a 2 ml polypropylene extraction tube (Ding-Guo Company, Beijing, China). Fenoterol (100 μl of 80 μg/ml solution) was added to each tube as the internal standard. Three hundred microliters of toluene was then added for extraction. This procedure was repeated three times. The merging extracts were evaporated with nitrogen stream in a water bath at 40 °C. The residue was then redissolved by 1.8 mM of NI containing 2% (v/v) acetonitrile for further chromatographic separation.

3. Results and discussion

3.1. Optimization of chromatographic conditions

The key work of this study is to achieve separation of the four β_2-agonists by using ion exchange chromatography with an aqueous solution as mobile phase. To achieve baseline separation, a series of experiments were performed to examine the effects of various factors on the separation.

3.1.1. Optimization of mobile phase

In this work, unsuppressed IC was used to separate the four β_2-agonists, the common eluents of unsuppressed IC being electrolyte solutions, such as diluted acidic solutions. Various acids at the same pH value (pH 2.98) were investigated as mobile phase, including nitric acid (NI), sulfuric acid (SU), phosphoric acid (PH), and citric acid (CI). The effect of the different acids upon the peak height and retention time of the four compounds is shown in Fig. 2. Fig. 2 illustrates that when NI was chosen as mobile phase, the peak height of each component reached the maximum value and the retention times of the four drugs reached the minimum value. Therefore, NI appears the most suitable eluent for the determination of the four agonists. We also investigated the effect of different acids upon the resolution (Rs) of the analytes, which is shown in Table 1. As can be seen in Table 1, NI is the most suitable acid as mobile phase.

In the preliminary experiments, we found that the peaks of the analytes, especially for clorprenaline and clenbuterol, were broad and the retention time of these two analytes were long. In order to improve the peak shape and obtain efficient separation, acetonitrile was added to modify the polarity of the mobile phase. It was found that peak broadening was

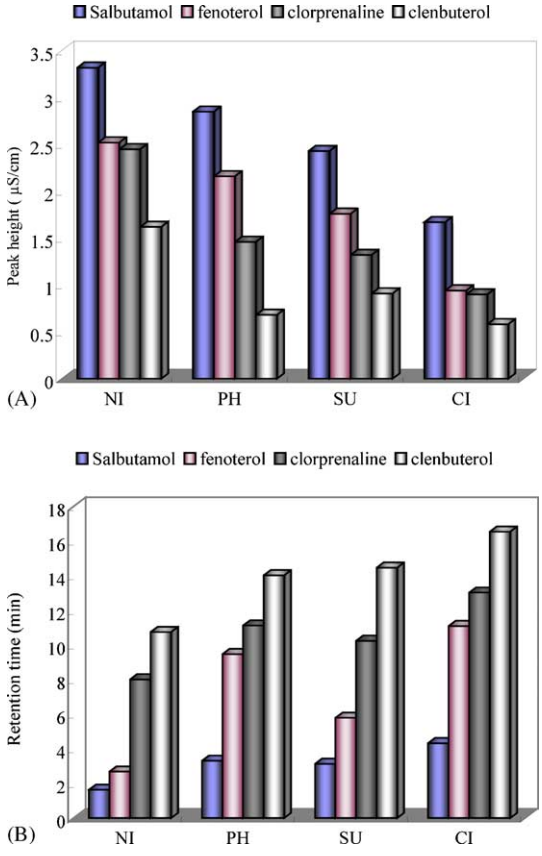

Fig. 2. Effect of a variety of acids on peak height (A) and retention time (B) of four drugs. NI: nitric acid; PH: phosphoric acid; SU: sulfuric acid; CI: citric acid. All acids are at pH 2.98. Chromatographic conditions: IC column, Metrosep cation1–2; sample: mixture of standard solutions; flow-rate: 1.0 ml/min; sample injection volume: 20 μl.

Table 1
Effect of different acids on the resolution of four β_2-agonists

Rs	NI	PH	SU	CI
Rs_{12}	1.12	1.98	0.63	1.79
Rs_{23}	2.55	0.71	2.12	0.67
Rs_{34}	1.01	0.60	0.57	0.73

(1) Salbutamol, (2) fenoterol, (3) clorprenaline, and (4) clenbuterol. NI: nitric acid; PH: phosphoric acid; SU: sulfuric acid; CI: citric acid. All acids are at pH 2.98. Chromatographic conditions: IC column, Metrosep cation1–2; sample: mixture of standard solutions; flow-rate: 1.0 ml/min; and sample injection volume: 20 μl.

greatly decreased by adding acetonitrile to the mobile phase. In order to obtain the best separation results, the effect of acetonitrile concentration was investigated. With a fixed NI concentration of 2.0 mM, the concentration of acetronitrile was changed from 2–8%. The acetonitrile concentration effects are illustrated in Fig. 3.

Fig. 3 illustrates that when the acetonitrile concentration is higher than 2% (v/v), the peaks of salbutamol and fenoterol overlapped, hence incomplete separation was achieved. A baseline separation could be reached when the acetonitrile concentration was 2% (v/v). Therefore, a 2% (v/v) acetonitrile concentration was applied for subsequent work.

The effect of NI concentration upon retention time and Rs was also studied. Fixing the concentration of acetronitrile at 2% (v/v), the concentration of NI was changed from 1.0 to 2.0 mM. The result showed that Rs between fenoterol and clorprenaline improved, but Rs between salbutamol and fenoterol, and Rs between colrprenaline and clenbuterol decreased when the NI concentration was altered from 1.0 to 2.0 mM. The retention time values are relatively long when the NI concentration was 1.0 mM and the analyte peaks were broad. Along with the increasing NI concentration, retention times of the four β_2-agonists decreased and peak shapes improved. The effect of NI concentration on the retention time was most obvious in the case of clenbuterol. Considering both Rs and retention time, with a concentration of 1.8 mM, clenbuterol can be detected within 8 min and all these β_2-agonists can be separated well. Therefore, the NI concentration was presently illustrated at 1.8 mM for further work.

Therefore, 1.8 mM nitric acid with a 2% (v/v) acetonitrile was used throughout the subsequent study.

3.1.2. Effect of mobile phase flow-rate

Flow-rate affects not only separation efficiency but also the peak shape. The retention times of the four analytes were examined at flow-rate of 0.6, 0.8, 1.0, 1.2 and 1.4 ml/min. When the flow-rate is 0.6 ml/min, the detection time obviously becomes relatively long, increasing flow-rates leading to shorter retention times apart from better peak shapes. However, with flow-rates higher than 1.0 ml/min, the Rs of the first two peaks decreased. In order to obtain efficient separation and adequate sensitivity, a 1.0 ml/min flow-rate was chosen in the following experiment.

The chromatogram illustrating the separation of the four β_2-agonists under the optimum conditions is shown in Fig. 4.

3.2. Linearity and detection limits

Under the optimum conditions described above, the detection limits were of 2.0 ng/ml for salbutamol, 10 ng/ml for fenoterol, 3.0 ng/ml for clorprenaline, and 10 ng/ml for

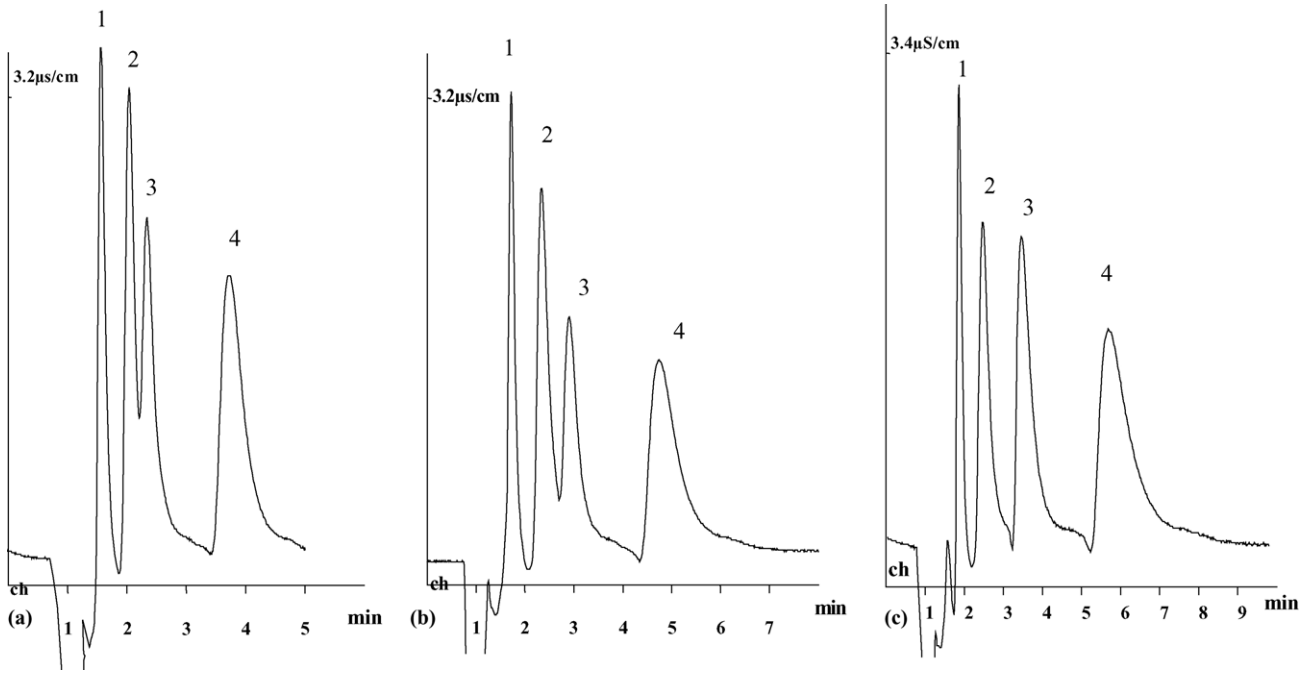

Fig. 3. Effect of acetonitrile concentration on retention time of four β_2-agonists in 2.0 mM nitric acid. (1) Salbutamol, (2) fenoterol, (3) clorprenaline, and (4) clenbuterol. Chromatographic conditions: IC column, Metrosep cation1–2; sample: mixture of standard solutions; flow-rate: 1.0 ml/min; sample injection volume: 20 μl. (a) Eight percent (v/v) acetonitrile concentration; (b) 4% (v/v) acetonitrile concentration; and (c) 2% (v/v) acetonitrile concentration.

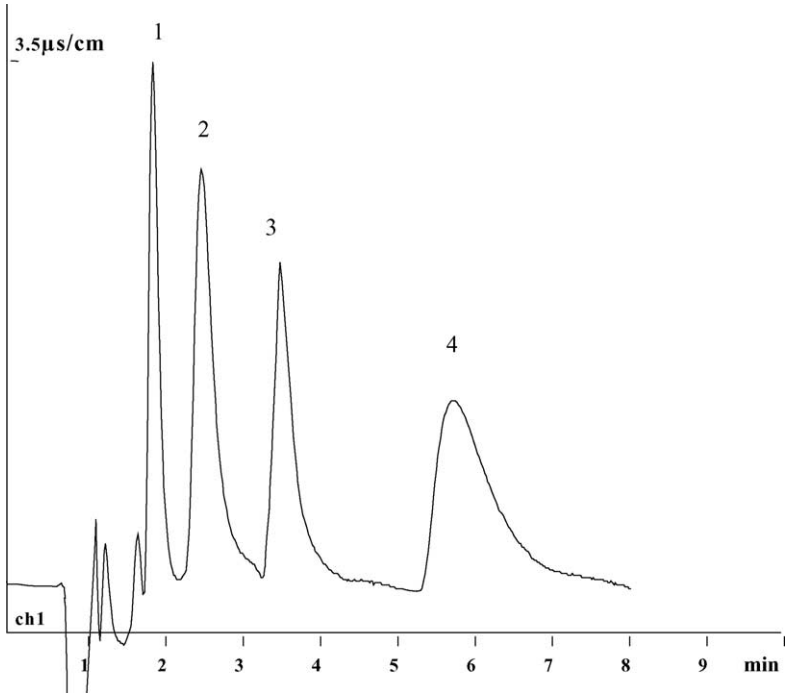

Fig. 4. Chromatogram for the separation of four β_2-agonists in 1.8 mM nitric acid and 2% (v/v) acetonitrile. (1) Salbutamol, (2) fenoterol, (3) clorprenaline, and (4) clenbuterol. Chromatographic conditions: IC column, Metrosep cation1–2; sample: mixture of standard solutions; flow-rate: 1.0 ml/min; sample injection volume: 20 μl.

clenbuterol. The detection limits were calculated from the peak heights of the analytes, appropriately diluted, and the noise height at a signal-to-noise ratio of three. As reported in [18], the detection limits of salbutamol and fenoterol obtained by HPLC methods with UV detection were 34.7 and 27.8 ng/ml. It is higher compared with the detection limits obtained by the method developed in our paper. The linear range was between 7.0 and 1.4×10^3 ng/ml for salbutamol, 34 and 7.8×10^3 ng/ml for fenoterol, 8.0 and 1.6×10^3 ng/ml for clorprenaline, and 25 and 7.5×10^3 ng/ml for clenbuterol. The regression coefficient (r^2) was 0.9959, 0.9970, 0.9935 and 0.9949 for salbutamol, fenoterol, clorprenaline, and clenbuterol, respectively.

3.3. Sample analysis

The contents of salbutamol and clenbuterol in tablets were analyzed using the proposed method under the optimum condition as described above. The tablets were homogeneously mortared to a fine powder and dissolved using the mobile phase, filtered, then injected into the sample loop for measurement. No clean-up procedure is involved. The β_2-agonists in the samples were identified by comparing their retention times with those of the standards. The contents of β_2-agonists in the samples were calculated by comparing peak area with those of the known standards. Recovery tests were carried out so as to evaluate if the present method is suitable for application to real samples. As shown in Table 2, the drug compounds can be well recovered from the tablets. The recoveries ranged from 97.2 to 100.4% and 101.0 to 104.1%. The method shows promise for the determination of pharmaceutical preparations.

The importance of determining salbutamol level in biological matrices for the investigation of banned substance in sport testing and growth promoter in animals is well recognized and the determination of salbutamol in plasma is important for clinical treatment. Salbutamol levels in plasma could also be detected in the present experiments. Due to the low con-

Table 2
Recovery of salbutamol and clenbuterol in tablets

Analyte	Content sample (μg/ml)	R.S.D. (%) (n = 11)	Added (μg/ml)	Total (μg/ml)	Recovery (%) (n = 3)
Salbutamol sulfate tablets®	0.0480	2.2	0.0500	0.0990	97.20
(approval number 282113)		2.0	0.0750	0.123	100.4
		1.8	0.100	0.148	99.20
Clenbuterol hydrochloride tablets®	0.200	1.3	0.250	0.458	104.1
(approval number 228301)		1.1	0.500	0.705	102.4
		2.8	0.750	0.952	101.0

Chromatographic conditions: IC column, Metrosep cation1–2; sample: mixture of standard solutions; flow-rate: 1.0 ml/min; and sample injection volume: 20 μl.

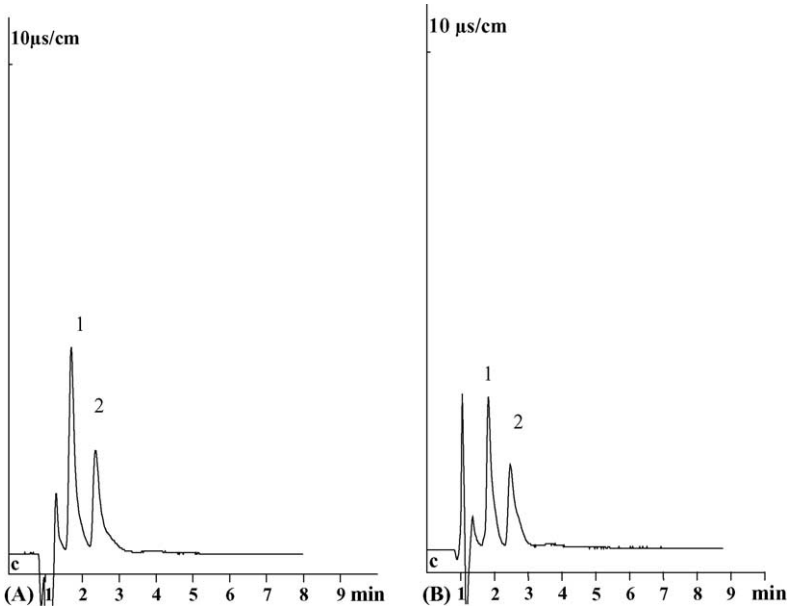

Fig. 5. (A) Chromatogram of human plasma extract spiked with salbutamol (10.0 ng/ml) and fenoterol (50.0 ng/ml); (B) chromatogram of human plasma 4 h after oral salbutamol dosing. (1) Salbutamol and (2) fenoterol.

Table 3
Intra- and inter-day variation, accuracy and extraction recoveries of salbutamol in plasma

Concentration (ng/ml)	Intra-day ($n = 5$)			Inter-day ($n = 5$)		
	Found (ng/ml)	R.S.D. (%)	Recovery (%)	Found (ng/ml)	R.S.D. (%)	Recovery (%)
10.0	9.8	2.7	98.0	9.6	3.6	96.0
50.0	49.3	5.2	98.6	51.2	4.8	102.4
500.0	506.3	5.2	101.3	502.3	5.8	100.5

centration of salbutamol in plasma, an extraction procedure is needed. The extraction procedure was cited in Section 2. Under the optimal conditions described above, we detected plasma samples spiked with salbutamol at 10.0 ng/ml and fenoterol at 50.0 ng/ml, and the samples after oral administration of salbutamol dosing with fenoterol as internal standard were also detected. Human plasma chromatograms are shown in Fig. 5. In order to validate the method, a recovery test was carried out on samples to which known amounts of salbutamol were added. The intra-day and inter-day precision and accuracy values were determined from analyzing plasma samples ($n = 5$) spiked with salbutamol at three concentrations; the result was summarized in Table 3. It can be observed that a satisfying recovery was achieved for salbutamol in plasma.

4. Conclusions

The β_2-agonists (salbutamol, fenoterol, clorprenaline, and clenbuterol) can be successfully separated and detected applying IC and the contents of salbutamol and clenbuterol in tablets can be analyzed by coupling IC with direct conductivity detection, allowing the determination of biologically important amines employing aqueous solution as mobile phase. The method was successfully applied to clinical samples from volunteers, clearly demonstrating that this new method is sensitive and applicable for the study of β_2-agonists in biological samples. The method described in this paper provides a rapid, simple, and sensitive procedure for routine control analysis of pharmaceutical preparation containing the cited β_2-agonists.

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