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Short communication

Quantitation of salbutamol in human urine by liquid chromatography–electrospray ionization mass spectrometry

Jianli Zhang [a,*], Youxuan Xu [a], Xin Di [b], Moutian Wu [a]

[a] *China Doping Control Center, National Research Institute of Sports Medicine, The State Sport General Administration, 1 Anding Road, Beijing 100029, China*
[b] *School of Pharmacy, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang 110016, China*

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Abstract

A sensitive and specific liquid chromatography–electrospray ionization-mass spectrometry (LC–ESI-MS) is described for quantitation of salbutamol in human urine using nadolol as the internal standard (I.S.). Urine samples were hydrolyzed with β-glucuronidase followed by a solid-phase extraction procedure using Bond Elut-Certify cartridges. The HPLC column was an Agilent Zorbax SB-C_{18} column. A mixture of 0.01 M ammonium formate buffer (pH 3.5)–acetonitrile (85:15, v/v) was used as the mobile phase. Analytes were quantitated using positive electrospray ionization in a quadrupole spectrometer. Selected ion monitoring (SIM) mode was used to monitor *m*/*z* 166 for salbutamol and *m*/*z* 310 for I.S. Good linearity was obtained in the range of 10.0–2000.0 ng/ml. The limit of quantification was 10.0 ng/ml. The intra- and inter-run precision, calculated from quality control (QC) samples was less than 7.3%. The accuracy as determined from QC samples was within ±2.6%. The method was applied for determining excretion curves of salbutamol.
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1. Introduction

Salbutamol is clinically the most widely used β_2-agonist in the treatment of bronchial asthma. The use of salbutamol in sports is forbidden by the oral route due to a strong adrenergic stimulation and an anabolic-like effect [1]. Salbutamol is excreted in urine as a mixture of the unchanged drug and its conjugated metabolite, mainly sulfate [2,3]. World Anti-Doping Agency (WADA) prescribes: when a concentration of salbutamol (free plus glucuronide) greater than 1000 ng/ml, this will be considered as an adverse analytical finding of anabolic agent [4]. Therefore, it is necessity in antidoping control to determine salbutamol in human urine.

In recent years, various assays have been developed to determine salbutamol in urine including GC–MS [5–8], HPLC-fluorimetric detection [9] and LC–MS methods [10–12]. Due to the presence of polar functional groups in the salbutamol molecules, a derivatization step is mandatory to convert the target analyte into compound suitable for gas chromatography analysis. This makes the procedure complex. And LC provides significant advantages over GC for the analysis of β_2-agonists, including compatibility of the biological matrix and the lack of any need for derivatization. HPLC-fluorimetric assay also is an alternative method for quantitation of salbutamol, but its lack of selectivity means that it is necessary to confirm positive results by MS techniques. Van Rhijn et al. [10] described a semi-quantitative method for the analysis of β_2-agonists by LC–MS–MS and the results of the analysis only yield semi-quantitative information, except for salbutamol. Van Vyncht et al. [11] developed a LC–MS–MS strategy for five β_2-agonists including salbutamol in biological matrix, but the quantification tests of salbutamol were performed in liver samples. Hogendoorn et al. [12] showed a rapid and sensitive LC–LC–MS–MS method which is able to determine the unconjugated salbutamol and the salbutamol conjugates was not investigated. In addition, the linearity ranges of these methods are not wide enough to be applied in doping control.

In this paper, we report a LC–ESI-MS method which provides sensitive and rapid quantitation of salbutamol in human urine to a lower limit of quantitation of 10.0 ng/ml using 1 ml of urine.

* Corresponding author.
E-mail address: zhang_jianli@sohu.com (J. Zhang).

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The assay is validated over the range of 10.0–2000.0 ng/ml and the method has been used successfully to determine excretion histograms of two volunteers (a female and a male).

2. Experimental

2.1. Chemicals and reagents

Salbutamol (for quantitation) was a gift from the National Institute for the Control of Pharmaceutical and Biological Products (China, Beijing). Nadolol (internal standard, I.S.) and β-glucuronidase (H-2) were purchased from Sigma Company (USA, Sigma). Salbutamol sulphate tablet preparation was supplied by Jiangsu Linhai Pharmaceutical Company (China, Beijing). Acetonitrile (HPLC grade) was obtained from Merck Company (Germany, Merck). Methanol and isopropyl alcohol (both of HPLC grade) were provided by Tedia Company (USA, Tedia). Sodium acetate anhydrous (analytical grade) was purchased from Wenzhou Chemical Reagent Company (China, Beijing). Twenty-five percent ammonia was obtained from Beijing Xinguang Chemical Company (China, Beijing). Ammonium formate (analytical grade) was purchased from Beijing Xizhong Chemical Company (China, Beijing). Formic acid, glacial acetic acid, ammonium chloride, chloroform (all of analytical grade) were supplied by Beijing Chemical Company (China, Beijing). Bond Elut-Certify solid phase extraction cartridges (300 mg/6 ml) were provided by Varian Company (China, Beijing). Deionised water was obtained by a purification system from Barnstead Company (USA).

2.2. Instrument and conditions

HPLC analyses were performed using an Agilent 1100 LC/DAD/MSD system (Agilent, USA) with a Zorbax SB-C_{18} column (150 mm × 2.1 mm, 5 μm; Agilent, USA). The mobile phase was 0.01 M ammonium formate (pH 3.5)–acetonitrile (85:15, v/v), the column temperature was maintained at room temperature. A constant flow-rate of 0.25 ml/min was employed throughout the analyses. LC–ESI-MS was carried out using nitrogen to assist nebulization. A quadrupole mass spectrometer equipped with an electrospray ionization source was used in positive ion selected ion monitoring (SIM) mode, set with a drying gas (N_2) flow of 10 l/min, nebulizer pressure of 35 psig, drying gas temperature of 300 °C and capillary voltage of 3 kV. The fragmentor voltage was 200 V. Target ions were monitored at *m*/*z* 166 for salbutamol and *m*/*z* 310 for I.S. in the SIM mode.

2.3. Preparation of standard solutions, calibration standards and quality control samples

Stock solutions of salbutamol and I.S. were prepared at 1 mg/ml in methanol, respectively and stored at 4 °C. Standard solutions containing 0.1, 0.5, 1, 5, 10 and 20 μg/ml salbutamol were prepared by diluting the stock solution with methanol. A I.S. working solution containing 5 μg/ml nadolol was also prepared using methanol.

Calibration standards of salbutamol (10.0, 50.0, 100.0, 500.0, 1000.0 and 2000.0 ng/ml) were prepared by spiking appropriate amount of the standard solutions in blank urine from healthy volunteers. Quality control (QC) samples were prepared in blank urine at concentrations of 10.0, 100.0 and 2000.0 ng/ml.

2.4. Sample preparation

A 1 ml urine sample was pipetted into a centrifuge tube and 40 μl of I.S. working solution were added. The urine was adjusted to pH 5.0 with 1.1 mol/l sodium acetate buffer (pH 5.0) and 40 μl of β-glucuronidase were added. The type of β-glucuronidase used in this study is H-2 which is from helix pomatia and can hydrolyze sulphate conjugate. The sample was vortex mixed, heated to 55 °C for 2 h on a water bath, and later cooled to room temperature. Urine sample was adjusted to pH 9.5 with ammonium chloride buffer (pH 9.5). After vortex mixing, the sample was centrifuged at 2500 rpm for 5 min. Bond Elut-Certify column was conditioned by washing with 2 ml of methanol and 2 ml of deionised water. The column was prevented from drying before applying specimens. Urine sample was applied to the pre-column. The column was washed consecutively with deionised water (2 ml), pH 4 acetic acid (1 ml) and methanol (2 ml). After drying for 2 min, two consecutive elutions (2 ml each, joint collection) were carried out with a mixture of chloroform-isopropyl alcohol (80:20, v/v) containing 2% ammonium hydroxide. The extracts were then evaporated to dryness under a stream of nitrogen at 40 °C. The residue was reconstituted in 200 μl of the mobile phase, and a 5 μl aliquot was injected into the LC–ESI-MS system.

2.5. Assay validation

2.5.1. Specificity

Specificity of the method was investigated by the analysis of three different sources of human urine including blank urine samples, blank urine samples spiked with salbutamol and I.S., and volunteer samples.

2.5.2. Linearity

Urine samples were quantified using the ratio of the peak area of salbutamol to that of I.S. as the assay parameter. Peak area ratios were plotted against concentrations and salbutamol concentrations were calculated using weighed ($1/x^2$) least squares linear regression.

2.5.3. Precision and accuracy

To evaluate linearity, urine calibration curves of each preparation procedure were prepared and assayed in triplicate on three separate days. Accuracy and precision were also assessed by determining QC samples at three concentration levels (Table 1, six samples each) on three different validation days. The accuracy was expressed by[(mean observed concentration)/(spiked concentration)] × 100% and the precision by relative standard deviation (R.S.D.).

Table 1
Precision and accuracy of the assay for determination of salbutamol in human urine ($n = 3$ day, six replicates per day)

Added (ng/ml)	Found (ng/ml)	Intra-run R.S.D. (%)	Inter-run R.S.D. (%)	Relative error (%)
10.0	10.1	7.0	7.3	1.4
100.0	102.6	5.3	5.8	2.6
2000.0	1951.6	6.2	6.5	−2.4

2.5.4. Extraction recovery

The recoveries of salbutamol at three QC levels were determined by comparing the peak area ratios of analyte to internal standard in samples that had been spiked with analyte prior to extraction with samples to which the analyte had been added post-extraction. The internal standards were added to both sets of samples post-extraction.

2.6. Application of the assay

Urine samples from two volunteers (a female and a male) were collected predose (blank urine) and after oral administration 2.4 mg of salbutamol sulfate at different collection periods. The obtained urine samples were stored at −20 °C until analysis. Before the excretion studies of salbutamol, the human experiments were approved by the ethical committee of national research institute of sports medicine, the state sport general administration. And volunteers have signed an informed consent before they participated in excretion studies.

3. Results and discussion

3.1. Conditions of chromatography

The selection of mobile phase components was a critical factor in achieving good chromatographic peak shape and resolution. A solvent system of ammonium formate and acetonitrile was selected as a buffer for its good volatility. Good separation of target compounds and short run time were obtained using an elution system of ammonium formate–acetonitrile (85:15, v/v).

3.2. Conditions for ESI-MS

The ESI mass spectrum at a fragmentor voltage of 100 V showed that the protonated molecular ion $[M + H]^+$ of salbutamol was at m/z 240. By increasing fragmentor voltage, the fragmentation pattern of this protonated molecular ion was observed. The product ion mass spectrum of this protonated molecular ion is shown in Fig. 1 (I) in which the most intense product ion was observed at m/z 166. By monitoring this product ion, a highly sensitive assay for salbutamol was developed. This intensity of

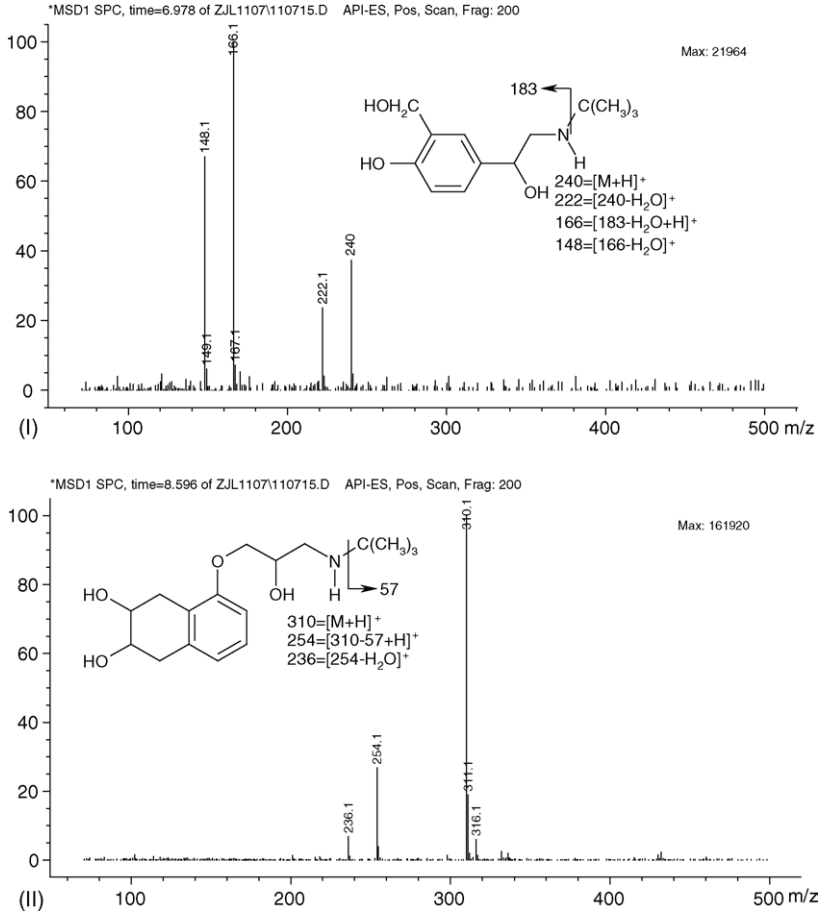

Fig. 1. Positive-ion ESI mass spectrum of salbutamol (I) and nadolol (II, I.S.) at 200 V.

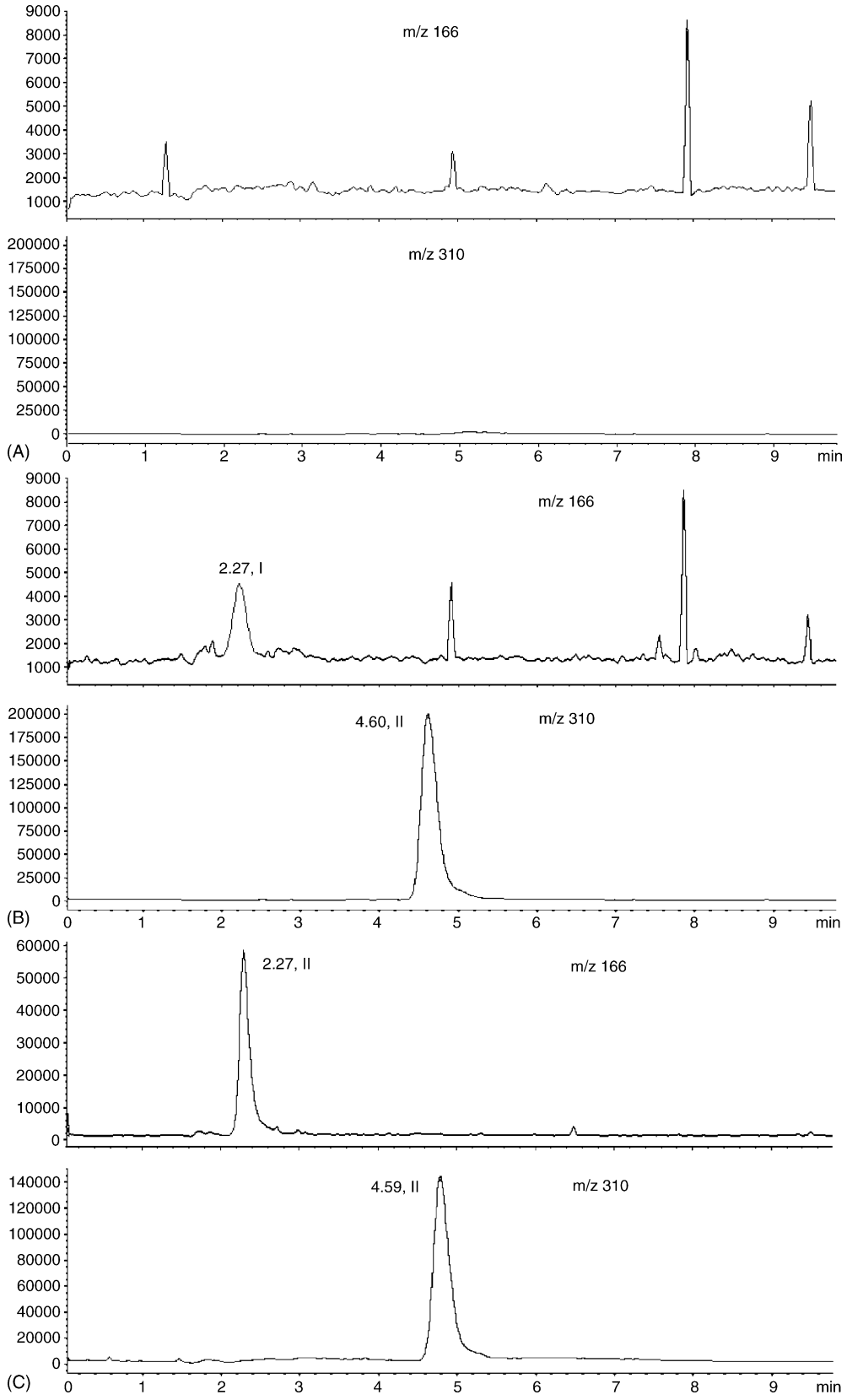

Fig. 2. Representative SIM chromatograms of (A) a blank urine, (B) a blank urine sample spiked with salbutamol (10 ng/ml) and the I.S. (100 ng/ml) and (C) a urine sample about 40 ng/ml from a volunteer after oral administration of 2.4 mg of salbutamol sulfate. Peak I, salbutamol; peak II, I.S.

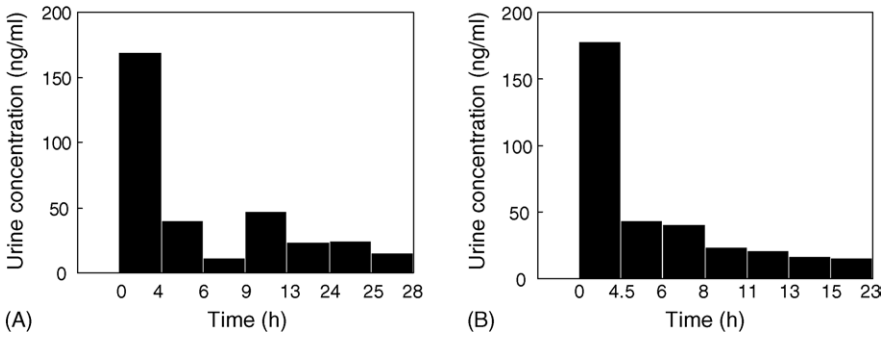

Fig. 3. Urine concentration–time column charts of (A) a female volunteer and (B) a male volunteer.

product ion of salbutamol at *m*/*z* 166 was compared at fragmentor voltages of 100, 150, 200, 250 and 300 V in order to determine the optimal collision energy. The result showed that the highest sensitivity was obtained using a fragmentor voltage of 200 V. Therefore, a fragmentor voltage of 200 V was used to carry out LC–ESI-MS in the SIM mode. At this collision energy the most intense product ion of I.S. protonated molecular ion was at *m*/*z* 310 (Fig. 1, II) which was selected as the target ion of I.S. in the SIM mode.

3.3. Method validation

3.3.1. Specificity

Potential interference from endogenous compounds was investigated. Representative chromatograms of a blank urine sample, a blank urine sample spiked with salbutamol at the LOQ and I.S., and a human urine sample after oral administration 2.4 mg salbutamol sulfate are shown in Fig. 2. No interferences from endogenous substances with analyte or I.S. were detected in blank urine.

3.3.2. Linearity and lower limit of quantitation

Visual inspection of the plotted triplicate calibration curves and correlation coefficients >0.99 confirmed that the calibration curves were linear over the concentration ranges 10.0–2000.0 ng/ml.

The limit of quantitation (LOQ) is defined as the lowest concentration on the calibration curve for which an acceptable accuracy of ±15% and a precision below 15% were obtained. The current assay had a lower limit of quantitation of 10.0 ng/ml.

3.3.3. Precision and accuracy

The method showed very good precision and accuracy. Table 1 summarize the intra- and inter-assay precision and accuracy for salbutamol from QC samples. The intra- and inter-assay precision were measured to be below 7.0% and 7.3%, respectively. The accuracy ranged from −2.4% to 2.6%.

3.3.4. Extraction recovery and storage stability

The recoveries of salbutamol at concentrations of 10.0, 100.0 and 2000.0 ng/ml were determined to be 71.3 ± 3.9% ($n = 6$) 75.1 ± 4.7% ($n = 6$) and 71.7 ± 6.9% ($n = 6$), respectively; and the extraction recoveries of I.S. were 80.7 ± 4.7% ($n = 6$) at concentrations of 100.0 ng/ml.

The stability of samples were determined from the low, medium and high QC urine samples by six replicates analyses of the three different concentrations (10.0, 100.0 and 2000.0 ng/ml). It was found that salbutamol in mobile phase was stable at least 12 h at room temperature. The REs for the three concentrations were ranged from −9.5% to −1.8%.

3.4. Application

The method described above was successfully applied to the excretion study of salbutamol for two volunteers (a female and a male). Urine samples were determined up to 28 h after administration of 2.4 mg of salbutamol sulfate as tablet. The urine concentration–time histograms are shown in Fig. 3.

4. Conclusion

This assay achieved high sensitivity and good specificity for analysis of salbutamol in human urine with a LOQ of 10.0 ng/ml. The calibration curve was linear over the range of 10.0–2000.0 ng/ml. This simple and rapid assay can be suitable used in antidoping control.

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