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# Salbutamol metabolism. How to differentiate oral vs. inhaled administrations: looking outside the box.

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## Abstract

Salbutamol is one of the most used $\beta_2$-adrenoceptor agonists by athletes for relieving bronchospasm and for prevention of exercise-induced-asthma and can be administered orally or by inhalation. Its use is approved by the World Anti-Doping Agency (WADA) only after inhaled administration. Previous studies have demonstrated that after oral administration, the majority of salbutamol is found in the urine either as the parent compound (24-33%) or as conjugated sulphate metabolite (48%). Enantioselective disposition studies have demonstrated that after oral administration the enantiomers are conjugated at a different rate by the body tissues. Therefore, after oral intake the non metabolized *S*(+) enantiomer is excreted in a greater level than the metabolized *R*(-) enantiomer.
The goal of this research was to develop an analytical method based on chiral chromatography tandem mass spectrometry for the simultaneous detection of salbutamol and its main metabolite. The applicability of the method was verified by the analyses of samples collected from healthy volunteers after the administration of salbutamol by different routes.
The method developed permitted the simultaneous detection of the separated enantiomers of the free and conjugated salbutamol in a single analytical run.
The preliminary results on real urinary samples confirmed that after inhalation the enantiomeric ratio between *S*(+) and *R*(-) of the non metabolized and of the metabolized salbutamol strongly depends on the percentage of the dose that is swallowed.

## Introduction

Inhaled administration of salbutamol is approved by WADA. If urinary concentrations are greater than 1200 ng/mL the non-inhaled administration must be excluded by other means [1].
Differential degree of sulphation of the enantiomers after oral intake [2-3] has been observed. In fact, the active R(-)-enantiomer undergoes a higher rate of sulphation, and therefore the ratio S(+)/R(-) and the S(+)+R(-) concentrations of free+glucuronide salbutamol have been proposed as markers to discriminate oral from inhalatory administrations [4]
We plan to develop a chiral HPLC/MS method for the simultaneous detection of free and sulphated salbutamol enantiomers in urine for its application in doping analyses, considering also the potential use of the sulphated fraction as a discriminating factor.

## Experimental

Chemicals and reagents
Salbutamol and salbutamol-D3 were supplied by Sigma-Aldrich (Milan, Italy) and Chemical Research (Rome, Italy) respectively. All chemicals were from Carlo Erba (Milano, Italy).

Excretion studies
Excretion studies were performed on 2 volunteers (1- male 40 years; 2- female 28 years) from which written consents were obtained. Both volunteers received Ventolin® (Glaxosmithkline S.p.A., Verona, Italy) at single dose of 2 mg p.o. and 200 µg by inhalation. Volunteer 2 received also a second 200 µg inhaled dose adding a washing mouth step to avoid swallowing part of the dose. After the administrations, urine samples were collected after 1 hour and then at 2 h time intervals for 8 h.

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Sample preparation
Three mL of urine spiked with salbutamol D3 (ISTD, 125 µg/mL urine final concentration) were extracted in an Oasis HLB cartridge (Waters, Milam, Italy) activated with 3 mL methanol and 3 mL water. The columns were washed with 1 mL of water and the analytes eluted with 3 mL methanol. The dried extracts were finally reconstituted with 150 µL of water or methanol or MPB.

Instrumental Conditions
The chromatographic separation was performed using an Agilent 1100 Series HPLC (Agilent Technologies S.p.a, Milano, Italy) and two columns for chiral separation Astec CHIROBIOTIC T (250 x 2.1 mm, 5 µm and 100x2.1 mm, 5 µm) from Supelco (Sigma-Aldrich, Milan, Italy) operating at 20 °C. The flow rate was set at 400 µL/min and 15 µL of sample extracts were injected. Different mobiles phases in isocratic conditions were investigated:
**- MPA:** Methanol/ acetic acid/ammonia; 1000: 5: 1 (v/v/v)
- **MPB**: Methanol/ acetonitrile/acetic acid/triethylammine; 800: 200: 1.5: 1 (v/v/v/v)
- **MPC**: Methanol/ acetonitrile/acetic acid/triethylammine; 700: 300: 0.25: 0.25 (v/v/v/v)

The mass spectrometer was an API4000 triple-quadrupole system (Monza, Italy). The MS conditions and specific selected monitoring reaction experiments are described in Table 1.

| Analyte | SRM Transition (m/z) | Collision energy | Mass spectrometric conditions |
|---|---|---|---|
| Salbutamol | 240/148<br>240/166<br>240/222 | 30 eV | MS: API4000 triple-quadrupole<br>ESI(+) mode<br>Ion source temperature: 500 °C<br>Capillary voltage : 5500 V<br>Declustering potential: 60 V |
| Salbutamol sulphate (salbutamol metabolite) | 320/148<br>320/240<br>320/222 | 30 eV | |
| Salbutamol d3 (internal standard) | 243/151<br>243/169<br>243/225 | 30 eV | |

Table 1. LC/MS instrumental conditions

## Results and Discussion

The main goal of the instrumental method was to analyse, on the same run, both free and sulphated salbutamol enantiomers. Although for the separation of free salbutamol enantiomers a CHIROBIOTIC T column of 100 mm was adequate, for the simultaneous separation of the 4-O-sulphate metabolite enantiomers the use of a 250 mm column was necessary.
Different mobiles phases were tested to achieve adequate enantiomeric resolution in a reasonable chromatographic time. The best chromatographic resolutions were obtained with MPC although with MPB the resolution was considered adequate with a shorter analytical run time as shown in Figure 1 (optimizations were performed with post-administration urine samples and elution orders were assigned based on literature data [5]).
Finally, different reconstitution solvents were tested (water, methanol and MPB). While the reconstitution of the final extracts with water did not permit the separation of the sulphated enantiomers, good separation and peak shape were obtained using methanol or MPB for both salbutamol enantiomers and their sulphates. Significant amounts of 4-O-sulphate were only detected after the oral administration. Opposite to the free salbutamol, the R(-)-sulphated salbutamol is excreted in higher amounts than the S(+) enantiomer.

As described in previous works [4], after the oral administration an increase of the S(+)/R(-) ratio is observed above the proposed discriminating value to disclose an oral from inhaled administration. Although a clear increase of the overall responses was observed after the oral administrations, only the S(+)/R(-) has been preliminary considered. In the inhaled

administrations, an increase is also observed since a relevant part of the dosage is swallowed. In fact, in the third excretion study with volunteer 2, where particular attention was taken to avoid swallowing salbutamol by including a mouth wash step, the S(+)/R(-) ratio was kept fairly constant, as expected (Figure 3).

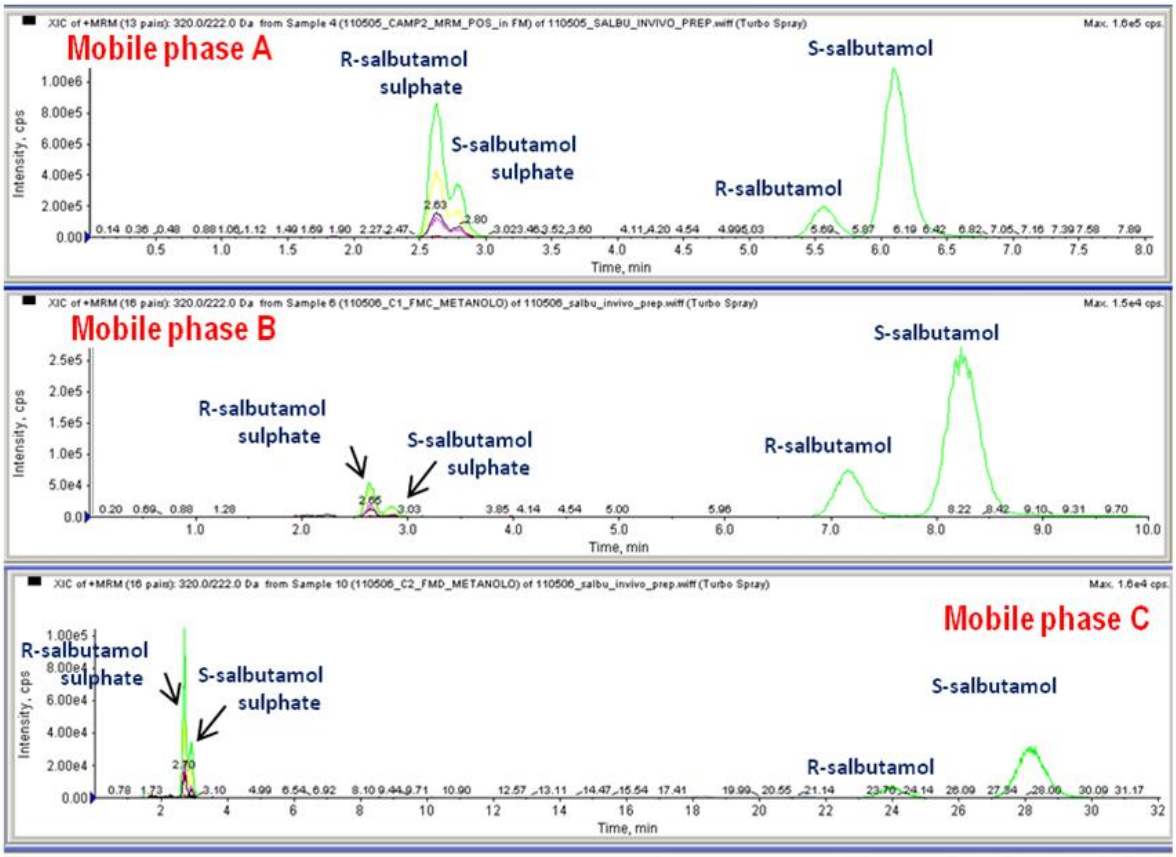


Figure 1: HPLC chiral separation optimization

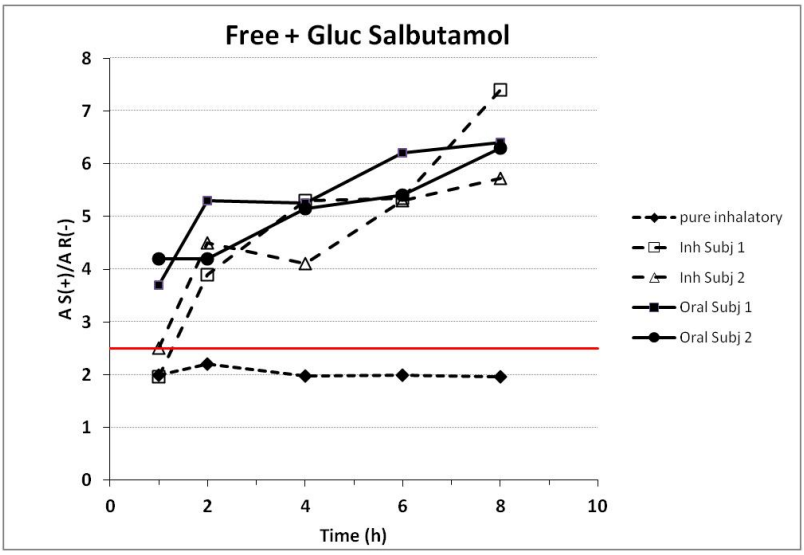


Figure 3. Time course of S(+)/R(-) ratio after salbutamol administration by different routes


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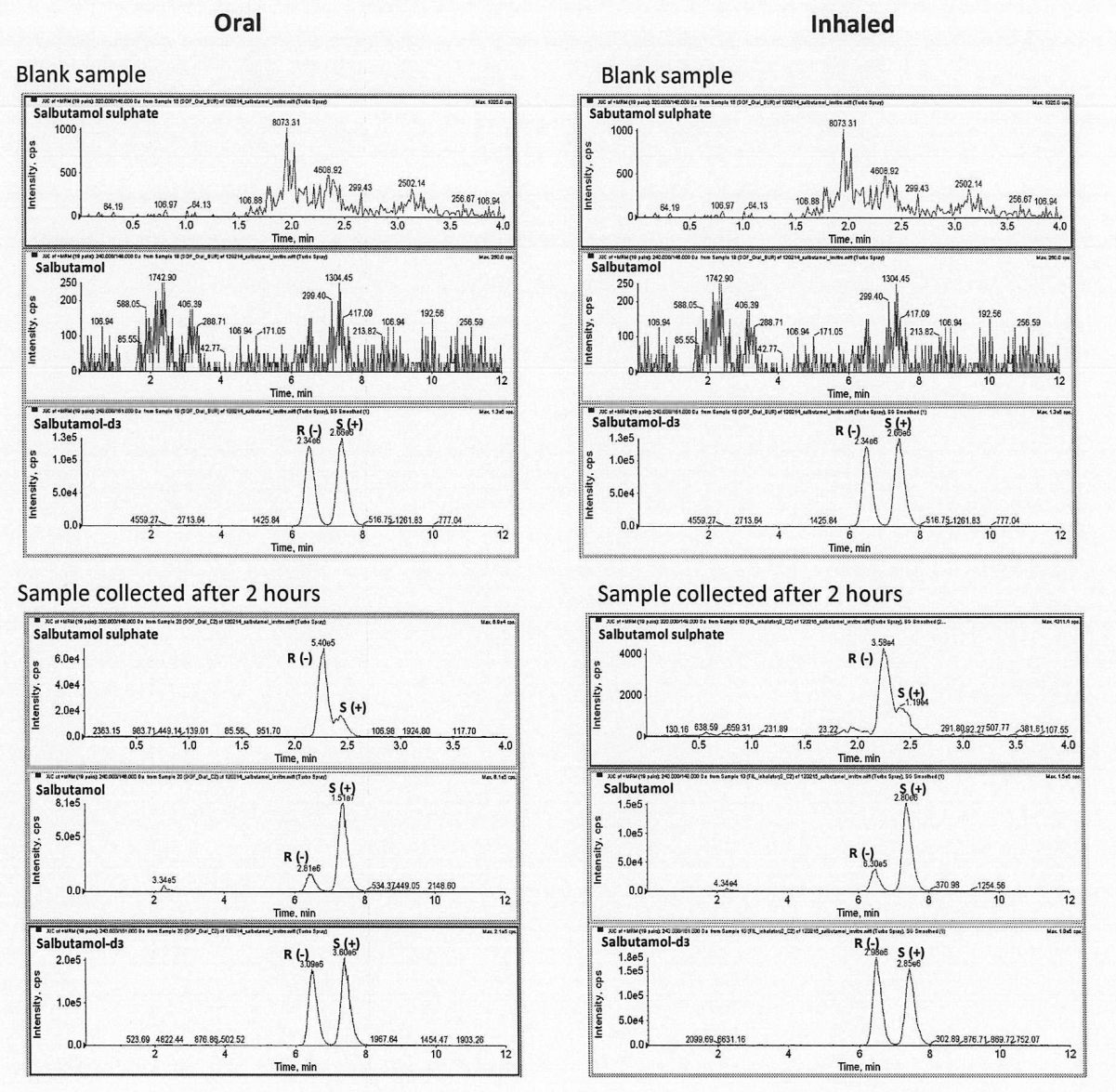


Figure 2. Detection of free and sulphated sabutamol after oral and inhaled administrations

## Conclusions

Good enantiomeric separation for free and sulphated salbutamol was achieved using the column CHIROBIOTIC (250x2.1 mm, 5 µm), the MPB and methanol as reconstitution solvent. With these conditions urine samples from excretion studies involving oral and inhaled salbutamol administrations were analyzed. Data obtained after pure inhalatory and oral administrations confirmed the criteria set by previous investigators (S (+)/R(-) ratio of 2.5) to discriminate oral from pure inhaled administrations. Mixed administrations (oral+inhaled) are still a challenge since the S(+)/R(-) ratio is strongly influenced by the swallowed dose, and because the free S(+)+R(-) value may be affected by the degree of sulphation. The number of volunteers studied does not permit to observe significant changes on the relative excretion of salbutamol-4-O-sulphate enantiomers.


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