

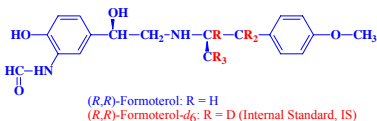
# Determination of (R,R)-Formoterol Inversion Products in Human Plasma and Urine By Chiral and Achiral-LC/MS/MS

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## Introduction

(R,R)-formoterol is a highly selective, potent, and long-acting  $\beta_2$ -adrenoceptor agonist currently under development for the maintenance treatment of asthma and the prevention of acute bronchospasm in patients with reversible obstructive airways disease. Formoterol contains two chiral centers with four diastereoisomers, i.e., (R,R)-, (R,S)-, (S,R)-, and (S,S)-formoterols. The (S,S)-formoterol is 1,000-fold less potent as a  $\beta$ -agonist than (R,R)-formoterol. In the current study, *in vivo* chiral inversion of (R,R)-formoterol to one or more of the other three stereoisomers was investigated. Four HPLC methods were established for separation of the four formoterol isomers in human plasma and urine samples. The HPLC effluents were monitored by liquid chromatography-tandem mass spectrometry (LC/MS/MS) with the limit of detection (LOD) at 1 or 3 pg/mL.



## Experimental

### Extraction Procedures:

#### Extraction Method 1 (for Chiral-HPLC/MS Method 1)

Human plasma or urine (1 mL) and IS-4 (100  $\mu$ L) were processed using a SPEC PLUS™ C18 cartridge. The desired eluate was evaporated to dryness under N<sub>2</sub> and the residue was reconstituted with 2 mL of 0.4% HCOOH, followed by partition with CH<sub>2</sub>Cl<sub>2</sub>. The aqueous fraction was evaporated to dryness and the residue was reconstituted with 50  $\mu$ L of CH<sub>3</sub>OH:CH<sub>2</sub>OH (1:1).

#### Extraction Method 2 (for Achiral-HPLC/MS Method 2)

Human plasma (1 mL) and IS-4 (100  $\mu$ L) were processed using a SPEC PLUS™ C18 cartridge. The desired eluate was evaporated to dryness under N<sub>2</sub> and the residue was reconstituted with 200  $\mu$ L of CH<sub>3</sub>OH:H<sub>2</sub>O (3:7).

#### Extraction Method 3 (for Achiral-HPLC/MS Method 3)

Human plasma (1 mL) and IS-2 (100  $\mu$ L) were processed using a SPEC PLUS™ C18 cartridge. The desired eluate was evaporated to dryness under N<sub>2</sub> and the residue was reconstituted with 200  $\mu$ L of CH<sub>3</sub>OH:CH<sub>2</sub>CN (4:6).

## Experimental (Cont.)

### Liquid chromatography:

LC System: Waters 2690 Separations Module or SHIMADZU Liquid Chromatography

#### HPLC Method 1 (Fractionation of four isomers)

Column: Chiralcel OJ-H, 5  $\mu$ m, 250x4.6 mm  
Mobile Phase: A: Hexane; B: Ethanol containing 1%DEA; C: CH<sub>3</sub>OH  
Isocratic: A:B:C (86:10:4)  
Flow Rate: 0.5 mL/min  
Injection Vol.: 15  $\mu$ L

#### HPLC Method 2 (Achiral, Acidic LC)

Column: Zorbax Eclips XDB-C18, 5  $\mu$ m, 2.1x50 mm  
Mobile Phase: A: 0.4% HCOOH in H<sub>2</sub>O; B: CH<sub>3</sub>OH  
Gradient: A:B=90:10 (0.5 min), 0.05 min to A:B=30:70 (2.45 min), 1 min to A:B=10:90 (0.05 min), 0.45 min to initial

Flow Rate: 0.3 mL/min (4 min), 0.05 min to 0.4 mL/min  
Injection Vol.: 35  $\mu$ L

#### HPLC Method 3 (Achiral, Basic LC)

Column: Phenomenex, Luna, 3  $\mu$ m, 100x4.6 mm  
Mobile Phase: A: 0.02%DEA in H<sub>2</sub>O; B: CH<sub>3</sub>CN  
Gradient: A:B=92:8 (2 min), 15 min to A:B=75:25 (5 min), 1 min to initial

Flow Rate: 0.4 mL/min  
Injection Vol.: 15  $\mu$ L

#### HPLC Method 4 (Chirabiotic-T, Chiral)

Column: Chiralbiotec-T, 250x4 mm  
Mobile Phase: A: CH<sub>3</sub>OH containing 0.045% HCOONH<sub>4</sub> and 0.1% HCOOH; B: Ethanol  
Isocratic: A:B (35:65)

Flow Rate: 0.6 mL/min  
Injection Vol.: 15  $\mu$ L

### Mass Spectrometry:

MS System: PE Sciex API 3000 tandem mass spectrometer coupled with Turbo-Ion Spray interface using multiple reaction monitoring (MRM) detection under positive ion mode

Ion Transitions: Formoterol:  $m/z$  345  $\rightarrow$   $m/z$  149  
Formoterol-*d*<sub>6</sub> (IS):  $m/z$  351  $\rightarrow$   $m/z$  155

## Results and Discussion

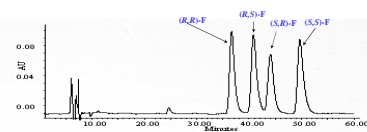


Figure 1. HPLC-UV Chromatogram of four formoterol isomers (HPLC Method 1)

1 Base-line separation of the four formoterol isomers was obtained under the normal phase chiral-HPLC conditions (Figure 1). The plasma and urine extracts (Extraction Procedure 1) were fractionated using the established HPLC method. The HPLC effluents were evaporated, reconstituted, and followed by LC/MS/MS analysis with a LOD at 0.5 pg/mL (Figure 2). The ion chromatograms were reconstructed using Microsoft® Excel. All four isomers were detected in the LOD sample at a concentration of 3 pg/mL (Figure 3). No inversion was observed in human plasma or urine collected from clinical trials (Figure 4).

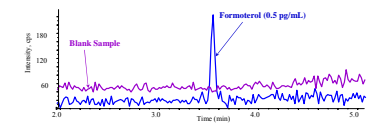


Figure 2. Mass ion chromatograms of a blank and a LOD sample (HPLC Method 2)

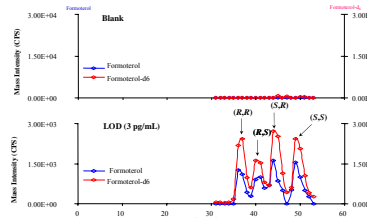


Figure 3. Reconstructed mass ion chromatograms of a blank and a LOD plasma sample (HPLC Method 1)

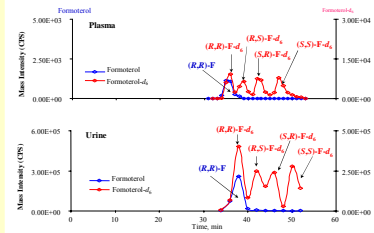


Figure 4. Reconstructed mass ion chromatograms of a human plasma and urine collected from a clinical trial (HPLC Method 1)

2 Although the four formoterol isomers eluted at a single peak under acidic HPLC conditions, the two pairs of enantiomers, i.e., (R,R)/(S,S)-formoterol and (R,S)/(S,R)-formoterol, were separated under HPLC basic conditions using a regular C<sub>18</sub> column. The HPLC effluents were monitored by LC/MS/MS with LOD at 1 pg/mL. No inversion of (R,R)-formoterol to (R,S)- or (S,R)-formoterol was observed in human plasma samples (Figure 5).

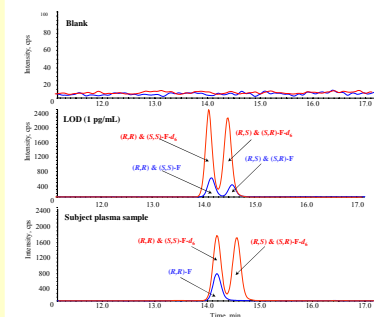


Figure 5. Mass ion chromatograms of a blank, a LOD (1 pg/mL), and a subject plasma sample (HPLC Method 3)

3 (R,R)-Formoterol and (S,S)-formoterol were separated using a Chirabiotic-T column (HPLC Method 4). The HPLC effluents were monitored by LC/MS/MS with LOD at 1 pg/mL. No inversion of (R,R)-formoterol to (S,S)-formoterol was observed in human plasma samples (Figure 6).

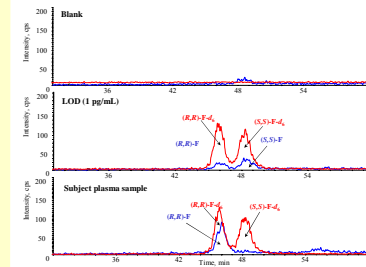


Figure 6. Mass ion chromatograms of a blank, a LOD, and a subject plasma sample (HPLC Method 4)

4 The separation and determination of four formoterol diastereoisomers can be achieved using HPLC Method 1. For large-scale sample analysis, the determination of possible inversion products can be obtained using a combination of HPLC Methods 3 and 4. The presence or absence of (R,S)- and (S,R)-formoterols can be determined using HPLC Method 3, and the presence or absence of (S,S)-formoterol can be determined by HPLC Method 4.

## Conclusions

- Three LC/MS methods were established for separation and determination of four formoterol diastereoisomers in human plasma and urine with a LOD at 1 or 3 pg/mL.
- No (R,R)-formoterol inversion products, i.e. (R,S)-, (S,R)-, or (S,S)-formoterol, were detected in human plasma or urine collected from clinical trials.

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