Monica Mazzarino, Ilaria Fiacco, Stefania Turi, Francesco Botrè

A simple and rapid procedure for the determination of the urinary concentration of formoterol by LC-MS/MS

Antidoping laboratory, Federazione Medico Sportiva, Rome, Italy

Abstract

The aim of this study was to determine the urinary excretion profile of formoterol and its metabolites after inhalation of different doses of two of the most used medicaments (Foradil® and Symbicort®) containing formoterol fumarate bihydrate. For this purpose a LC-ESI-MS/MS procedure for the quantification of formoterol in human urine was developed and validated. Sample preparation includes an enzymatic hydrolysis and a dilution step. Detection of analytes was performed by a triple quadrupole mass spectrometer under positive ion electro-spray ionization conditions and selected reaction monitoring acquisition mode. Good linearity (R^2 > 0.990), precision (not exceeding 10% and 15%, respectively for intra-day and inter-day assays), accuracy (below 15%), specificity and reproducibility of relative retention times (CV% < 1) and of relative abundances of characteristic ion transitions (CV% < 10) were obtained. The lower limits of detection and quantification were 1 and 2 ng/mL respectively.
Our results also show that the urinary profile of formoterol (40-60%) and its metabolites (O-demethylated metabolite 5-25%; glucuronide metabolites 25-40%) varies significantly depending on the different available drugs and the subject tested. Formoterol reaches the maximum after 1-3 hours from the administration. The maximum urinary levels measured in this study was 15 ng/mL (free+glucuronide), thus confirming that the WADA threshold, set at 30 ng/mL, is unlikely to be exceeded following the administration of therapeutic doses by the inhalatory route of administration.

Introduction

Since 1992, beta-2-agonists are included in the list of prohibited substances and methods, presently reviewed and updated by the World Anti-Doping Agency. In 2012 formoterol, a long-acting beta2-agonist, was removed from Section 3 of the WADA list of banned compounds and methods, when taken by inhalation at therapeutic dosages [1]. Consequently, an adverse analytical finding is issued only in the case the measured urinary concentration exceeds the established decision limit (38 ng/mL) [2]. We have developed and validated a LC-ESI-MS/MS procedure for the direct determination of formoterol in urine. The developed procedure was used to measure the urinary levels of formoterol after inhalation of different doses of medicaments (Foradil® and Symbicort®) containing formoterol fumarate bihydrate.

Experimental

Chemicals and reagents

Formoterol fumarate and $^{13}C,^{2}H_3$-formoterol hemifumarate (internal standard) were supplied by Alsachim (Illkirch, France). All chemicals were from Carlo Erba (Milano, Italy).
Excretion studies were performed on 4 males (30, 34, 35 and 43 years; 75, 70, 65 and 72 Kg) and 4 females (27, 36, 40 and 28 years; 55, 60, 50 and 55 Kg). Written consents were obtained from patients allowing the use of urines for research purposes. A dose of 12 or 36 µg of Foradil® (12 µg formoterol fumarate bihydrate, Novartis Farma S.p.A., Varese, Italy) or of 9 or 27 µg of Symbicort® (9 µg formoterol fumarate bihydrate, AstraZeneca, Milano, Italy) was administered twice a day (9:00 a.m. and 3:00 p.m.) for two days. The urines were collected after 1 hour and then every three hours from the drug(s) administration for 48 hours.

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Analytical procedure

The chromatographic separation was performed using an Agilent 1200 Rapid Resolution Series HPLC pump (Agilent Technologies S.p.a, Milano, Italy), a Discovery® C18 (150 X 2.1 mm, 5 µm) column from Supelco (Milano, Italy) and 0.1% of formic (A) and acetonitrile (B) as mobile phases. The gradient program starts at 2% of B and increases to 40% of B in 8 min and after 2 min to 80% of B for 1 min. The flow rate was 250 µL/min.

The mass spectrometry was an API4000 triple-quadrupole system (Monza, Italy) with positive electrospray ionization. The mass parameters and the selected ion transitions were reported in Table 1.

Sample pre-treatment includes an enzymatic hydrolysis (5 µL of ß-glucuronidase from *E. coli* and 100 µL of phosphate buffer, 1 M, pH 7.4) of 500 µL of urine for 1 hour at 50 °C. 5 µL of the hydrolyzed urine was then diluted with 200 µL of the ISTD (50 ng/mL). 20 µL were injected.

Compound	Ion transitions (m/z)	CE (eV)	T (°C)	DP (V)	Capillary voltage (V)	RRt
Formoterol	345/327; 345/149; 345/121	20; 30; 30	500	60	5500	1.0
Formoterol O-demethylted	331/313; 331/149; 331/121	20; 30; 30	500	60	5500	0.7
$^{13}C,^{2}H_3$-formoterol	349/153; 349/121	30; 35	500	60	5500	1.0

Table 1. Diagnostic ion transitions (selected reaction monitoring, SRM), collision energy, source temperature (T), declustering potential (DP), Applied capillary voltage and relative retention time (RRt)

Results and Discussion

The optimal mass spectrometry conditions were obtained by infusion of formoterol standard solution at a concentration of 10 µg/mL (Table 1). For the chromatographic conditions different stationary phases (C8, C18 and HILIC), mobile phase additives (formic and acetic acid, ammonium formate and acetate) and column temperature (30, 40 and 60 °C) were tested. The best conditions in term of reproducibility, sensitivity, peak shape, selectivity and chromatographic retention were obtained using the parameters reported in the experimental section (Figure 1). The optimized procedure was validated according to ISO 17025 and WADA requirements [2-4]. Good reproducibility of the relative retention times (CV% < 0.5) and of relative abundances of selected ion transitions (CV% < 10) were measured.

No significant interferences and matrix effect (< 10%) were detected at the retention times of formoterol and internal standard in more than 50 urines. The method was linear in the range of 2-60 ng/mL (R^2 > 0.990). The lower limit of detection and quantification were 1 and 2 ng/mL respectively. The accuracy was below 15% for all the three levels tested (10, 30 and 60 ng/mL) using ten replicate each. Good precision was obtained (10% for intra-day and 15% for inter-day assays) at the concentration levels tested (10, 30 and 60 ng/mL) using ten replicate each. The urinary excretion of formoterol (40-60%) and its metabolites (O-demethylated metabolite 5-25%; glucuronide metabolites 25-40%) vary significantly between the different available drugs and subject tested according to previous publications [5]. Figures 2A-B show the profiles of formoterol and its metabolite (free+glucuronide) after inhalatory administration of therapeutic and high doses of Foradil® and Symbicort®. Formoterol reach the maximum after 1-3 hours from the administration. The highest urinary levels (13-15 ng/mL) was measured in two females (subject 1 and 2) after administration of 36 µg of Foradil®.

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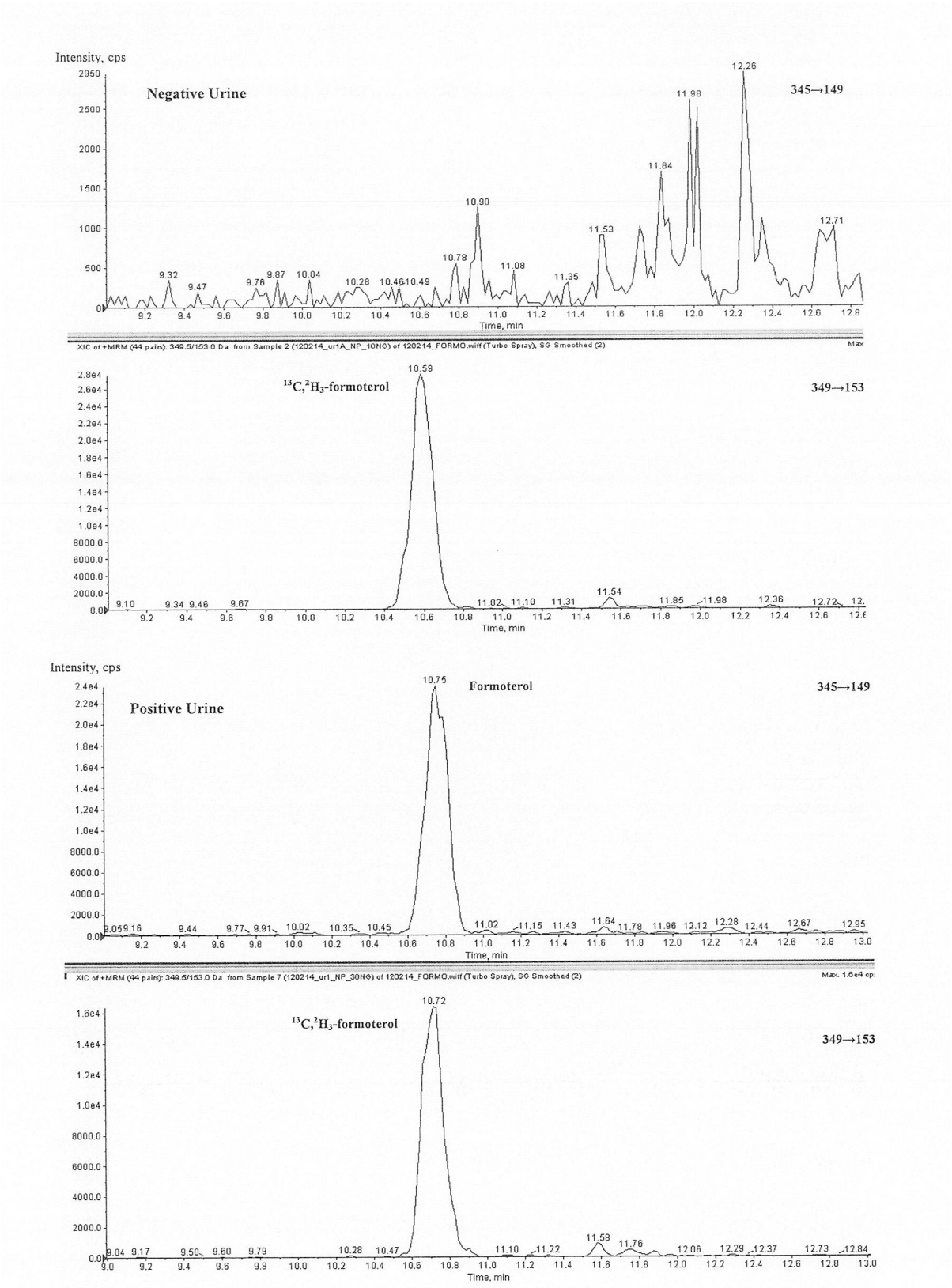

Figure 1. Extract ion chromatograms (XIC) of the LC-MS/MS analyses of a negative urine and a negative urine spiked with formoterol at a concentration of 30 ng/mL using the analytical procedure set up in this study.

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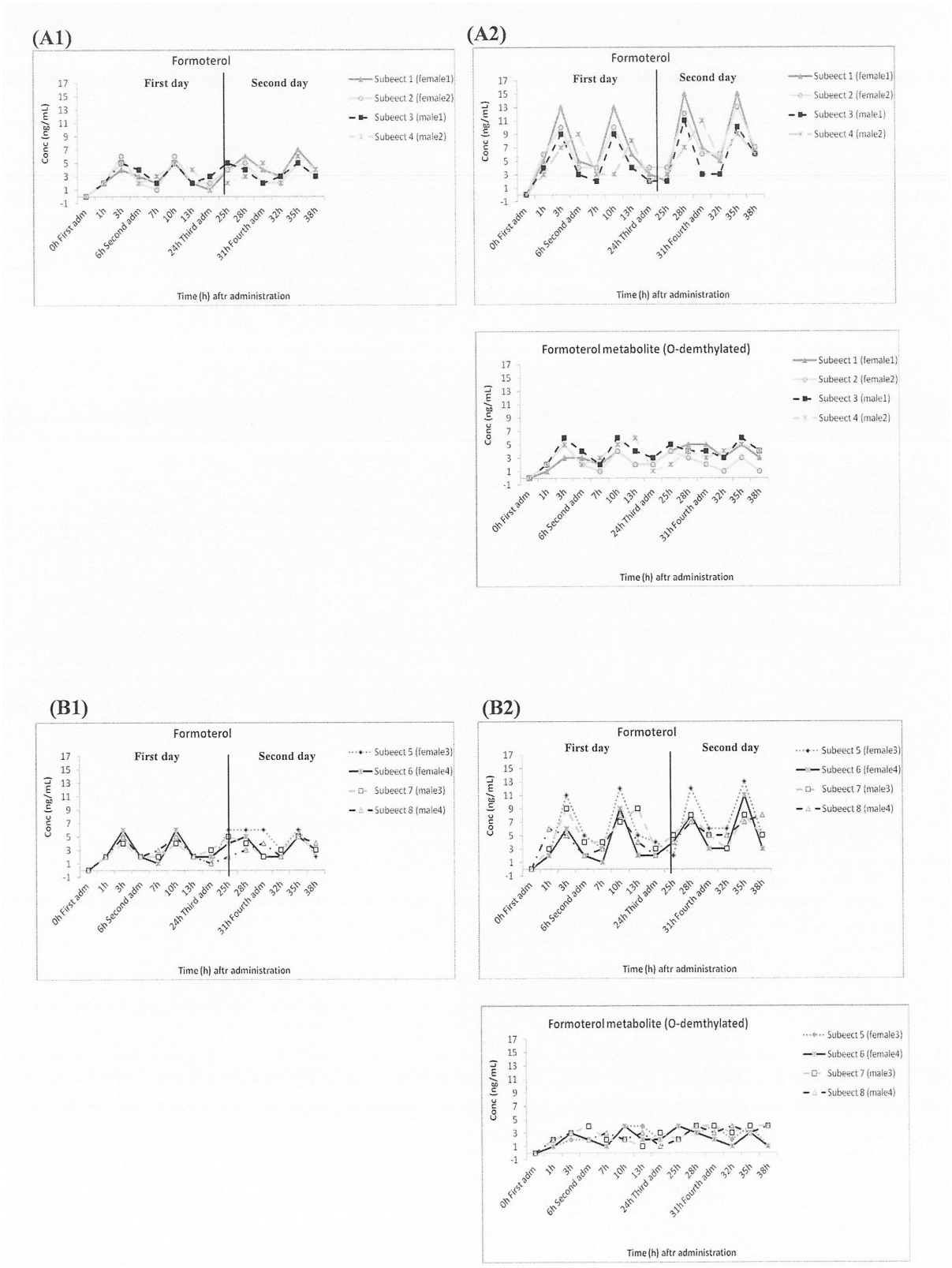

Figure 2. Urinary profiles of formoterol (free+glucuronide) and its O-demethylated metabolite (free+glucuronide) obtained after administration of therapeutic (A1, B1) and high dosages (A2, B2) of Foradil® (A) and Symbicort® (B). The demethylated metabolite was not detected after administration of therapeutic doses of the two drugs considered in this study.

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Conclusions

The data obtained demonstrate the capability and suitability of the direct LC-ESI-MS/MS analysis for the quantitative confirmation of formoterol in urine samples. The analytical procedure was fully validated, tested on real samples and showed comparable analytical performances with respect to LC-ESI-MS/MS procedures currently used in our laboratory, while reducing the analysis time and the human resources by a factor of 2.
The maximum urinary levels of formoterol measured after inhalation of high dosages of either Symbycort® or Foradil® were in all subjects tested below 20 ng/mL, according with previous publications [5]. Hence, it seems extremely unlikely that the threshold value (30 ng/mL) fixed by the WADA be exceeded by inhalation, even in the case of non therapeutic dosages.

References

[1] World Anti-Doping Agency. The 2012 Prohibited List. International Standard, Montreal (2012) http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/WADA_Prohibited_List_2012_EN.pdf (access date 20.06.2012).
[2] World Anti Doping Agency. Decision limits for the confirmatory quantification of threshold substances, (WADA Technical Document TD 2010IDL) Available: http://www.wada-ama.org.
[3] International Organization for Standardization, General requirements for the competence of testing and calibration laboratories, ISO:17025.
[4] World Anti Doping Agency. Identification criteria for qualitative assays incorporating column chromatography and mass spectrometry, (WADA Technical Document TD 2010IDCR) Available: http://www.wada-ama.org (access date 20.06.2012).
[5] Deventer K, Pozo OJ, Van Eenoo P, Delbeke FT. (2010) Excretion study with b2-agonists. In: Schänzer W, Geyer H, Gotzmann A, Mareck U. (eds.) *Recent Advances in Doping Analysis (20)*, Köln, pp 130-139.

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