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Evaluation of the urinary threshold concentration of formoterol in sports drug testing

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The use of formoterol in sports is allowed by inhalation at the maximum recommended therapeutic dose. Recently, a threshold concentration of 30 ng.mL^{-1} was defined by the World Anti-Doping Agency (WADA) to distinguish between therapeutic and forbidden use of formoterol. The objective of this work was to evaluate that threshold concentration. Concentrations of formoterol were measured in urine samples collected after administration of 18 μg of inhaled formoterol to five healthy volunteers, and in samples collected in routine doping tests belonging to athletes having declared inhaled formoterol use. Formoterol was detected up to 8 h after administration in all volunteers with concentrations up to 19.6 ng.mL^{-1}. From 28 routine samples, 27 had less than 10 ng.mL^{-1} of formoterol and only in one of the samples the concentration was 25 ng.mL^{-1}. Therefore, administration of formoterol by inhalation at the maximum dose allowed by WADA will not produce false positive results using a threshold concentration of 30 ng.mL^{-1}, and the experience up to now in routine doping tests indicates that the probability of obtaining urines with concentrations greater than 30 ng.mL^{-1} is close to nil. For this reason, sports authorities should re-evaluate the need of a threshold concentration for formoterol and its practical usefulness. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: sports testing; formoterol; threshold concentration; doping; mass spectrometry; urine

Introduction

Formoterol is a long-acting β_2-adrenoreceptor agonist used for the treatment of bronchial asthma, prevention of exercise-induced bronchospasm, and for chronic obstructive pulmonary disease (COPD).[1–3] It is formulated as a fumarate salt, and consists of a racemic mixture of two enantiomers. It is normally administered by inhalation either alone or in combination with a glucocorticosteroid.[1–4] Formoterol has an extended duration of action (up to 12 h) compared to short-acting β_2 agonists such as salbutamol.[1,2]

In addition to the desired pharmacological action, there is some evidence indicating that systemic β_2-agonists may have a positive effect on physical performance in healthy subjects[5] and, for this reason, its use in sports is restricted by the World Anti-Doping Agency (WADA). All β_2-agonists are prohibited with the exception of salbutamol, formoterol, and salmeterol when taken by inhalation in accordance to the manufacturer's recommended therapeutic regime.[6]

Since 2012, a maximum inhaled daily dose has been established for formoterol in the WADA prohibited list (maximum 36 μg over 24 h, according to the recommended therapeutic regime this dose corresponds to 18 μg every 12 h).[6] Besides, in an attempt to distinguish between therapeutic and forbidden administrations, a threshold concentration of 30 ng.mL^{-1} for formoterol has been established by WADA. The presence in urine of formoterol in excess of 30 ng.mL^{-1} is presumed not to be an intended therapeutic use of the substance and has to be considered as an adverse analytical finding unless the athlete proves, through a controlled pharmacokinetic study, that the abnormal result was the consequence of the use of the therapeutic inhaled dose up to the maximum.[6]

The definition of a threshold concentration requires the performance of different administration studies in healthy subjects to assess the urinary levels of the compound after administration of allowed doses.[7–10] In spite of the high number of pharmacokinetic studies of formoterol in healthy subjects[11–14] or patients with asthma or COPD,[15] few data on urinary concentrations of formoterol after inhaled administrations are available in the literature to support the threshold value defined by WADA.[16–19]

The objective of this work was to evaluate the threshold concentration of formoterol recently defined by WADA by studying urinary concentrations of the compound in healthy volunteers

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after inhalation of the maximum allowed dose and in samples from athletes with declared inhaled administration.

Experimental

Chemical and reagents

Formoterol fumarate was supplied by Novartis Pharma AG (Basel, Switzerland) and penbutolol sulfate by Hoechst Iberica (Barcelona, Spain). Methanol and 2-propanol (HPLC grade), glacial acetic acid, 25% ammonia, ammonium chloride, sodium acetate trihydrate, potassium hydroxide (analytical grade) were purchased from Merck (Darmstadt, Germany). Chloroform (HPLC grade) was supplied by Scharlau (Barcelona, Spain). Deionized water was obtained by a Milli-Q purification system (Millipore Ibérica, Barcelona, Spain).

β-Glucuronidase from *Helix pomatia* type HP-2 (Sigma Chemicals, St Louis, MO, USA), was used for enzymatic hydrolysis. *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA), gas chromatographic grade, was obtained from Macherey-Nagel (Düren, Germany). Bond-Elut Certify™solid phase extraction columns (130 mg/10 ml) were provided by Varian International (Harbor City, CA, USA).

Sample preparation

Analysis of formoterol in urine was performed using a procedure previously described.[16,20] Briefly, urine samples (5 ml) were added with the ISTD solution (60 µl of penbutolol 1 µg.mL^{-1}). Samples were adjusted to pH 5.2 with 1.1 mol.L^{-1} acetate buffer (pH 5.2) and 50 µL of β-Glucuronidase from *Helix pomatia* were added. After incubation (55°C for 2 h), samples were adjusted to pH 9.5 with ammonium chloride buffer (600 µl) and subjected to a solid-phase extraction with columns previously conditioned with methanol (2 ml) and deionized water (2 ml). Columns were washed with deionized water (2 ml), 0.1 mol.L^{-1} acetate buffer pH 4 (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/2-propanol (80:20, *v/v*) with 2% ammonium hydroxide. The organic phase was evaporated to dryness under a stream of nitrogen in a bath at 40°C, and the residues were maintained under vacuum for at least 30 min. The residues were then dissolved in 50 µL of MSTFA, vortex mixed and incubated at 60°C for 20 min.

Instrumental analysis

The extracts were analyzed by gas chromatography-mass spectrometry (GC-MS) using an Agilent 6890 gas chromatograph coupled to a 5973 mass-selective detector and a 7683 injector (Agilent Technologies, Palo Alto, CA, USA), and equipped with a crosslinked methyl siloxane fused-silica capillary column (17.5 m x 0.2 mm i.d., 0.11µm film thickness) (Agilent Technologies, Palo Alto, CA, USA). Injections were made in splitless mode using helium as carrier gas. Injector and transfer line temperatures were set to 280°C. Oven temperatures were programmed as follows: initial temperature 100°C for 2 min., increase at 30°C.min^{-1} to 190°C, increase at 20°C.min^{-1} to 320°C, maintained for 3 min. Sample injection volume was 1 µl. The analyses were performed using electron impact ionization and selective ion monitoring (SIM) mode. Three characteristic ions of formoterol (*m/z* 178, 277 and 367) and penbutolol (*m/z* 57, 86 and 348) derivatives were monitored. Ion *m/z* 178 was used for quantitation of formoterol.

Excretion study samples

Excretion studies involving the administration of formoterol to five healthy volunteers (two male, three female) and urine collection were performed following a protocol approved by the local Ethical Committee (Barcelona, Spain). A single inhaled dose of two puffs of 12 µg of formoterol fumarate (corresponding to 18 µg of formoterol) (Foradil® Aerosol, Novartis Pharma AG, Basel, Switzerland) was administered to each volunteer. Urine samples were collected before administratiom and up to 24 h after administration at the following collection periods: 0–1, 1–2, 2–4, 4–8, and 8–24 h. Samples were stored at -20°C until analysis.

Samples from atletes

Samples obtained in routine doping control belonging to athlets that declared the administration of inhaled formoterol were also analyzed (n = 28). Only samples with estimated formoterol concentration greater than 1 ng.mL-1 were selected.

Results and discussion

Formoterol is primarily metabolized by glucuronidation, at either the phenolic or aliphatic hydroxyl group, and by O-demethylation followed by glucuronide conjugation, at both phenolic hydroxyl groups. Minor pathways involve sulfate conjugation of formoterol and deformylation followed by sulfate conjugation.[21] Main metabolites in urine are free formoterol, and the conjugates with glucuronic acid of formoterol (mainly at the phenolic group) and O-demethylated formoterol. Consequently, enzymatic hydrolysis with an enzyme with β-glucuronidase and arylsulphatase activity was used in the sample preparation procedure in order to recover formoterol excreted free as well as the excreted conjugated with glucuronic acid or sulfate.

Concentrations of formoterol in urine were quantified using a method previously described.[16,20] Selectivity and specificity were demonstrated by the absence of interferent peaks at the retention times of formoterol and the ISTD in ten blank urine samples. Identification capacity of the method was demonstrated in a previous publication.[16] Determination coefficients greater than 0.99 were always obtained over the range from 0.5 to 50 ng.mL^{-1}. The limit of quantitation was 0.5 ng.mL^{-1} and the extraction recoveries for formoterol and for the ISTD were 87.1 ± 6.3% and 78.5 ± 6.4%, respectively. Precision and accuracy in intra- and inter-assay experiments (Table 1) were in the accepted range.

Concentrations of formoterol in samples collected after administration of a single inhaled dose of 18 µg of formoterol to five healthy volunteers are listed in Table 2. Formoterol was detected in urine for up to 8 h in all volunteers and in urines collected from 8 to 24 h for some of them. Concentrations ranged from 0.5 to 19.6 ng.mL^{-1}, depending on the collection period. The differences in concentrations between volunteers can be explained by taking urine dilution into account. More uniform data between volunteers were obtained using excretion rates. The maximum excretion rate was obtained for all volunteers from 1–2 h after administration of the compound (12.3 ± 1.8 µg/min). Results of concentrations after inhaled administration agree with previously published data.[16–19] After single inhaled doses of 18 µg of formoterol, concentrations in the range of 0.5–17 ng.mL^{-1},[16] up to

Table 1. Intra- and inter-assay precisions and accuracies obtained in the quantification of formoterol in urine in quality control samples (QC) at three diferent concentrations. RSD, relative standard deviation; RE, relative error

QC ($ng.mL^{-1}$)	Assay	Estimated concentration (mean ± sd) ($ng.mL^{-1}$)	Intra-assay Precision (RSD, %) (n = 3)	Intra-assay Accuracy (RE, %) (n = 3)	Inter-assay Precision (RSD,%) (n = 9)	Inter-assay Accuracy (RE,%) (n = 9)
1	1	1.0 ± 0.1	8.0	5.6	13.7	14.2
	2	1.2 ± 0.1	7.1	22.8		
	3	1.1 ± 0.2	16.3	11.3		
12	1	13.4 ± 0.9	7.1	11.3	8.7	12.0
	2	13.5 ± 1.2	8.9	12.7		
	3	13.4 ± 1.8	13.3	12.1		
20	1	20.9 ± 1.1	16.3	4.4	7.1	8.3
	2	21.0 ± 1.6	13.3	7.5		
	3	19.3 ± 1.4	7.4	6.6		

Table 2. Concentrations of formoterol detected in urine after administration of inhaled formoterol fumarate to five healthy volunteers

Time (h)	VOL1[a] ($ng.mL^{-1}$)	VOL2 ($ng.mL^{-1}$)	VOL3 ($ng.mL^{-1}$)	VOL4 ($ng.mL^{-1}$)	VOL5 ($ng.mL^{-1}$)	mean ± s.d. ($ng.mL^{-1}$)
pre-dose	-	-	-	-	-	
0–1	0.8	2.1	14.6	0.7	10.9	5.8 ± 6.5
1–2	5.0	10.4	19.6	1.7	3.5	8.1 ± 7.2
2–4	10.1	10.4	10.2	1.7	2.8	7.0 ± 4.4
4–8	0.5	2.3	1.1	1.4	0.5	1.2 ± 0.7
8–24	< LOQ[b]	1.2	< LOQ	1.3	-	0.6 ± 0.6

[a]VOL: volunteer
[b]LOQ: limit of quantification

11.4 $ng.mL^{-1}$[18] and up to 15.9 $ng.mL^{-1}$[19] were obtained in previous studies. After repeated inhaled doses, concentrations up to 15 $ng.mL^{-1}$[17] and 25.6 $ng.mL^{-1}$[19] were described. The cumulative percentage of the dose recovered in urine up to 24 h ranged from 10.7 to 17.9% (mean 14.7%), in agreement with data of previous studies.[12,13,15,18]

It is worth to notice that 8 h after administration, only residual concentrations of formoterol were detected in urine (Table 2), indicating that no accumulation of the compound occurs. In agreement with our results, concentrations of formoterol the third day after repeated inhaled doses did not significantly differ with concentrations obtained the first day of administration.[17] The inhaled dose applied in our study was 18 μg of formoterol which is half of the maximum dose in 24 h allowed by WADA (36 μg over 24 h). According to the recommended therapeutic regime, a second dose of 18 μg can be inhaled 12 h after the first administration to obtain the maximum dose allowed. Because, there is no accumulation of formoterol, the second administration will result in concentrations in urine similar to those obtained after the first dose. Therefore, the concentration of 30 $ng.mL^{-1}$ will be hardly reached. In summary, administration by inhalation of the maximum dose of formoterol allowed by WADA following the recommended therapeutic regime should not produce false positive results using the threshold concentration of 30 $ng.mL^{-1}$. In a recent study,[19] administrations every 2 h of 18 μg of inhaled formoterol resulted in concentrations in urine up to 21.4 $ng.mL^{-1}$ after the second dose (total dose, 36 μg) and up to 25.6 $ng.mL^{-1}$ after the fourth dose (total dose, 72 μg). Therefore, the results obtained up to now, support the threshold concentration defined by WADA even at higher doses than the recommended therapeutic regime.

Concentrations of formoterol detected in urine samples obtained from routine doping controls are shown in Figure 1. Only samples with concentrations greater than 1 ng/ml were selected. These samples belong to sportsmen having declared the inhaled administration of formoterol. In most of the samples, concentrations below 10 $ng.mL^{-1}$ were found. Only one sample with 25 $ng.mL^{-1}$ of formoterol was seen. Similar results were obtained by Deventer *et al.*[18] where a maximum concentration of 20.8 $ng.mL^{-1}$ was observed in 82 routine doping samples containing formoterol.

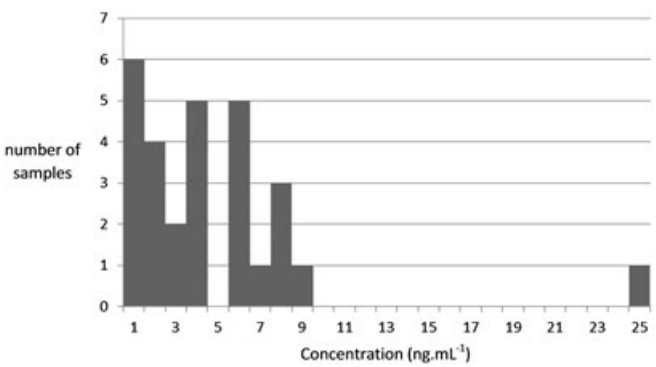

Figure 1. Distribution of formoterol concentrations ($ng.mL^{-1}$) in routine samples (n = 28).

The restriction of the use of formoterol in sports is due to its potential effect on physical performance when administered by systemic routes. However, in most countries, formoterol is only commercialized in preparations to be used by inhaled administration.[22] So, its systemic administration is unlikely. In addition, as it is shown in this paper, the experience of our laboratory in routine doping samples containing formoterol and the experience recently reported by other doping control laboratories[18] indicate that the probability of obtaining urines with concentrations greater than 30 ng.mL^{-1} in routine tests is close to nil. Therefore, according to these data, laboratories will never need to apply the threshold concentration in routine samples. For these reasons, the sense of the threshold concentration for formoterol is unclear.

In conclusion, sports authorities should re-evaluate the need and the practical usefulness of a threshold concentration for formoterol using all the data available up to now regarding urinary concentrations of the compound after therapeutic administrations and the concentrations detected in routine doping tests.

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