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Excretion studies with beta-2-agonists

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Introduction

Beta-2-agonists have a pronounced and very effective bronchodilatating effect [1]. Consequently they are amongst the most prescribed drugs for humans in the treatment of asthma. Besides the desired pharmacological action some β_2-agonists produce, at higher doses, side-effects on protein synthesis and lipolysis resulting in anabolic action [2]. Hence β_2-agonists might be misused in sports for stimulatory effect on respiration and the central nervous system and growth-promoting action when administered in higher doses. To control the use of β_2-agonists, the World Anti Doping Agency (WADA) put them on the list of prohibited substances [3]. However, because of their frequent therapeutic application the use is allowed when administered by inhalation. The condition of use is that the athlete should possess a declaration of use [4]. Consequently, β_2-agonists are frequently declared on the doping control forms [5] and are frequently detected during routine doping-analysis.

In the past, several papers describing the detection of β_2-agonists by GC-MS and LC-MS were published [2, 6-8]. Because limited information is available regarding urinary concentrations of β_2-agonists after inhalation [9-12], it is difficult to assign the detected concentrations in the routine samples to therapeutic use or to doping misuse. The objective of this study was to investigate urinary concentrations of β_2-agonists after inhalation.

Experimental

Chemicals and reagents

Salmeterol was obtained from Glaxo-Welcome (Brussels, Belgium) and salmeterol-d_3 from CDN-Isotopes (Quebec, Canada). Formoterol was obtained from Boehringer Ingelheim (Brussels, Belgium) and formoterol-d_6 from Medical Isotopes (Pelham, USA). Terbutaline was obtained from Astra-Zeneca (Brussels, Belgium) and terbutaline-d_9 from Medical Isotopes (Pelham, USA). Acetic acid (HOAc) p.a., sodium acetate (NaAc) p.a., isopropanol,

diethyl ether, potassiumcarbonate (K_2CO_3), disodiumhydrogencarbonate ($NaHCO_3$) and sodiumhydroxide (NaOH) were of analytical grade and were purchased from Merck (Darmstadt, Germany). Methanol (MeOH), ammonium acetate (NH_4Ac) and HPLC grade water were from Biosolve (Valkenswaard, The Netherlands). Beta-glucuronidase (containing aryl-sulphatase activity) from *Helix Pomatia was* from Sigma-Aldrich (Bornem, Belgium). Buffer pH 5.2 was obtained by dissolving 136 g Na-acetaat into 800 ml of aqua bidest. The pH was adjusted to 5.2 by adding actic-acid. Then the final volume was made to 1L. Buffer pH 9.5 was prepared by dissolving 45g K_2CO_3 and 37g NA_2HCO_3 in 300 mL of H_2O

Excretion studies

The study was performed with 6 male healthy volunteers aged 23, 28, 29, 31, 34 and 39. The study protocol was reviewed and approved by the ethical committee of the Ghent University Hospital (UZGent, Project B67020072141). Each volunteer signed a statement of informed consent. The administered doses are presented in table 1.

Table 1: Product, administration route and administered dose

Product	Administration route
Terbutaline (Bricanyl ®)	Aërosol (500 µg)
Formoterol (Oxis ®)	Aërosol (18 µg)
Salmeterol (Serevent ®)	Aërosol (100µg)

Urine samples were collected before (0h) and quantitatively at 1, 2, 3, 6, 9, 12 hours after intake. Additional samples were collected after 24, 36, 48 and 72h. All urine samples were stored at -20°C awaiting analysis. Volume and density were measured and all samples were analyzed in duplicate.

Sample treatment

Salmeterol and formoterol

Fifty microliter of an internal standard solution (100 ng/mL of the deuterated analogue) was added to 1 mL of urine, followed by the addition of 1 mL of acetate bufferr (pH 5.2) and 50 µl of enzyme from helix Pomatia. After 2.5h of incubation at 56°C, 1 ml of carbonate buffer pH 9.5 was added. Liquid-liquid extraction was performed by rolling for 5 min with 5 mL diethylether/isopropanol (1/1). After centrifugation (1.5 g) the organic layer was transferred into a new tube and evaporated until dry under oxygen free nitrogen (OFN) at 40°C.

Terbutaline

Terbutaline has a phenolic hydroxy group in meta position. The presence of this group makes terbutaline susceptible to the pictet-spengler reaction wich occurs at low pH and in the presence of formaldehyde [13].

When terbutaline undergoes the reaction a so called, tetrahydroisoquinoline derivate (THIQ) is formed. Despite its toxicity, formaldehyde is excreted in urine in the range of 2-4 µg/mL.[14]. Since the enzymatic hydrolysis step is conducted at a low pH (pH 5.2) at elevated temperature it is very likely that terbutaline is converted during sample preparation. The results of an incubation experiment shows that terbutaline is converted into the THIQ derivate when exposed to formaldehyde under the enzymatic hydrolysing conditions.

Since the amounts of formaldehyde in real samples can differ from the amounts in the urine used for constructing the calibration curve quantification can be erroneous. Hence to have a correct quantification it was decided to first convert quantitatively the present terbutaline into the THIQ-derivate. Therefore a conversion step was included after the enzymatic hydrolysis.

The sample preparation is as follows.

An internal standard solution (50 µL, 100 ng/mL of terbutaline-d_9) was added to 1 mL of urine, followed by the addition of 1 mL of acetate buffer (pH 5.2) and 50 µl of enzyme from helix Pomatia.. After 2.5h of incubation at 56°C, 25 µl of formaldehyde 0,37% was added and the sample was incubated overnight at 80°C. After cooling to room temperature 1 ml of carbonate buffer pH 9.5 was added. Liquid-liquid extraction was performed by rolling for 5 min with 5 mL diethylether/isopropanol (1/1). After centrifugation (1.5 g) the organic layer was transferred into a new tube and evaporated until dry under oxygen free nitrogen (OFN) at room temperature.

Apparatus

The HPLC system consisted of a Surveyor MS-pump and Surveyor autosampler with a 50 µL sample loop (Thermo Separation Products, Thermo, San Jose, CA, USA).

Separation was performed on a Zorbax RX C8-colulmn (150x2mm, 5 µm) from Agilent (Diegem, Belgium). The column was maintained at 35ºC. The mobile phase consisted of water (A) and MeOH (B), both containing 1 mM NH_4Ac and 0,1% HAc. Gradient elution at a flow rate of 0.4 mL/min was performed as follows: 100% A for 0.5 min decreased to 50% A in 4.5 min and further decreased to 5% of A in 0.5 min. Isocratic for 1 min followed by an increase to the initial condition of 100% A in 0.1 min followed by an equilibration step of 2.3 min before the next injection. Total analysis time per sample was 9 min. The LC effluent

was pumped to a Quantum Discovery mass spectrometer (Thermo, San Jose, CA, USA) equipped with an ESI source, operated in the positive ionisation mode. The capillary temperature was 350 ºC. The sheath gas flow rate was set to 50 arbitrary units. No auxilliary gas was used. The quantifier ions are presented in Table 2.

Table 2: SRM-settings for the investigated substances.

Substance	PI/QI/CE/TLV	Substance	PI/QI/CE/TLV
Salmeterol	416/232/22/123	Salmeterol-d_3	419/91/50/123
Formoterol	345/149/19/123	Formoterol-d_6	351/155/19/123
Terbutaline- THIQ	238/162/35/100	Terbutaline-d_9 THIQ	247/165/20/100

PI: precursor ion, QI: quantifier ion, CE: collision energy, TLV: tube lense voltage

Validation

A six-point calibration curve was generated by spiking blank urine with the respective beta-2-agonists in triplicate at 0.5, 1, 5, 10, 25 and 50 ng/mL. The ratio of target compound product ion area to IS product ion area were plotted versus concentration to obtain calibration curves. Precision and accuracy were tested at the lowest, middle and highest calibrator. Precision was assessed as the percentage relative standard deviation (%RSD) of both repeatability (within-day) and reproducibility (between-day and different analysts) for a selected level. Maximum allowed tolerances for reproducibility and repeatability can be calculated from the Horwitz-equation $RSD_{max} = 2^{(1-0.5\log C)}$ (C = concentration (µg/mL) x 10^{-6}). Maximum allowed tolerances for repeatability and reproducibility were 2/3 RSD_{max} and RSD_{max}, respectively [15]. Inaccuracy was defined as the difference between the calculated amount and the specified amount as a percentage [16]. The limit of quantification (LOQ) of the method was defined as the lowest concentration where acceptable reproducibility and accuracy could be guaranteed.

The limit of detection (LOD) was defined as the lowest concentration with a signal to noise ratio (S/N) higher than 3. Selectivity was tested by analysing several structurally related and other routinely screened doping agents, including corticosteroids, anabolic steroids, diuretics, stimulants, narcotics and beta-blocking agents. Specificity was tested by analysing 6 blank urines as described to evaluate the presence of endogenous interferences.

Evaluation of the ion suppression was achieved by extracting 6 blank urines following the aforementioned procedure without the addition of IS-solution. After evaporating the organic solvent, the remaining residues were spiked with 50 µL of the internal standard solution and with the respective beta-2-agonist at 10 ng/mL. The ion suppression was then determined by comparing the areas in the urine samples with a water sample spiked at the same concentration.

Results and discussion

Validation results

Using a least square fit, good linearity ($r^2 \geq 0.98$) was observed for all 3 compounds. The results for imprecision and inaccuracy are summarised in Table 3 and did not exceed 2/3 RSD_{max} or RSD_{max} neither for repeatability nor reproducibility. Deviation of the mean measured concentration from the theoretical concentration (inaccuracy) was below the acceptable threshold of 15% and 20 % for all levels in the range of the calibration curve [16]. Regarding the selectivity, interferences from other β_2-agonists and other doping agents could not be found. In addition analysis of 10 different blank control urine samples did not result in the detection of interfering substances, proving the specificity of the method. The limit of quantification (LOQ) of the method was 0.5 ng/mL.

Table 3: Bias and imprecision (RSD) for repeatability and reproducibility and tolerance limits.

Substance	Conc (ng/mL)	Repeatability Bias (%)	Reproducibility Bias (%)	Repeatability RSD (%)	Reproducibility RSD (%)	RSD_{max} (%)	$2/3RSD_{max}$ (%)
Salmeterol	0.5	4.5	10.4	12.3	8.6	48	32
	10	-3.1	4.9	5.2	6.2	32	21
	50	-3.7	-2.9	2.2	4.2	25	17
Formoterol	0.5	-1.0	-6.8	17.1	13.3	48	32
	10	-0.8	-2.9	1.8	2.0	32	21
	50	-0.05	-0.3	1.6	1.0	25	16
Terbutaline	0.5	-7.6	0.6	8.4	10	48	32
	10	0.04	-3.8	3.8	9.2	32	21
	50	-1.9	-3.0	5.7	8.4	25	16

Excretion study

Salmeterol

For all volunteers salmeterol could be detected already 1 hour after intake.

The maximum urinary concentration was reached between 1 and 3 hour for all volunteers. The peak concentrations ranged between 0.1 ng/ml and 1.27 ng/ml.

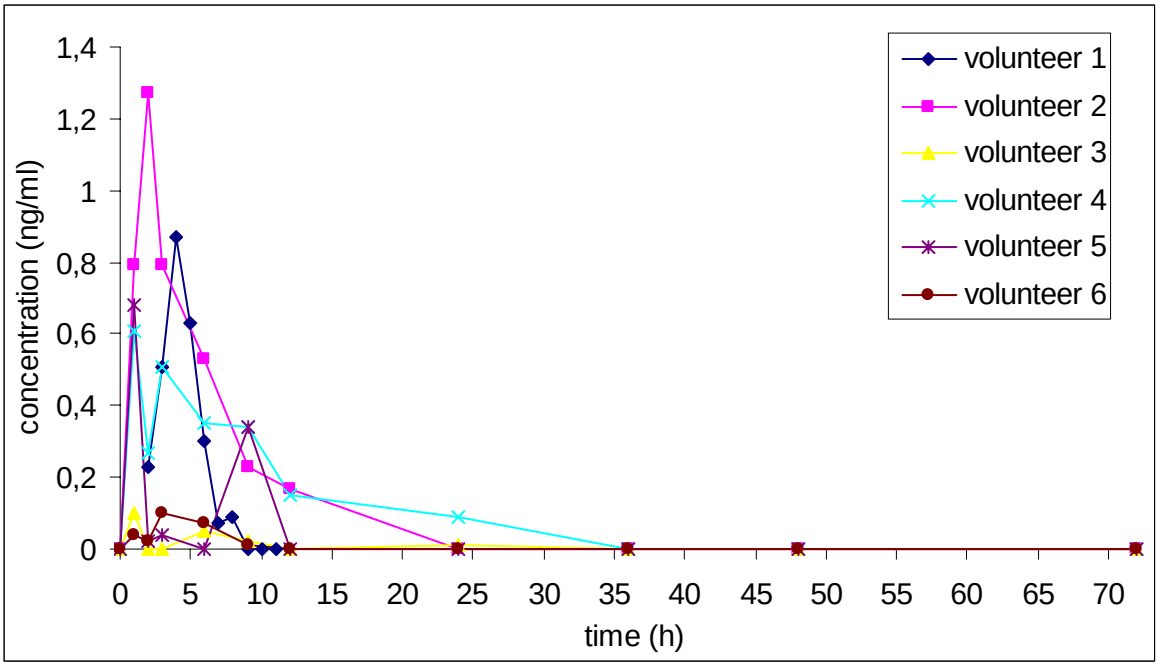

Figure 1: Concentrationprofile of excreted salmeterol.

Detection times ranged up to 48 hours for some volunteers. The total amount of unchanged drug excreted during the first 12 hours varied between 92 and 298 ng, corresponding to 0.04 and 0.23% of the administered dose.

Formoterol

The excretion profiles are presented in figure 2. For all volunteers formoterol could be detected already 1 hour after intake. The maximum urinary concentration was reached between 1 and 3 hour for all volunteers. The peak concentrations ranged between 2.3 and 11.4 ng/ml.

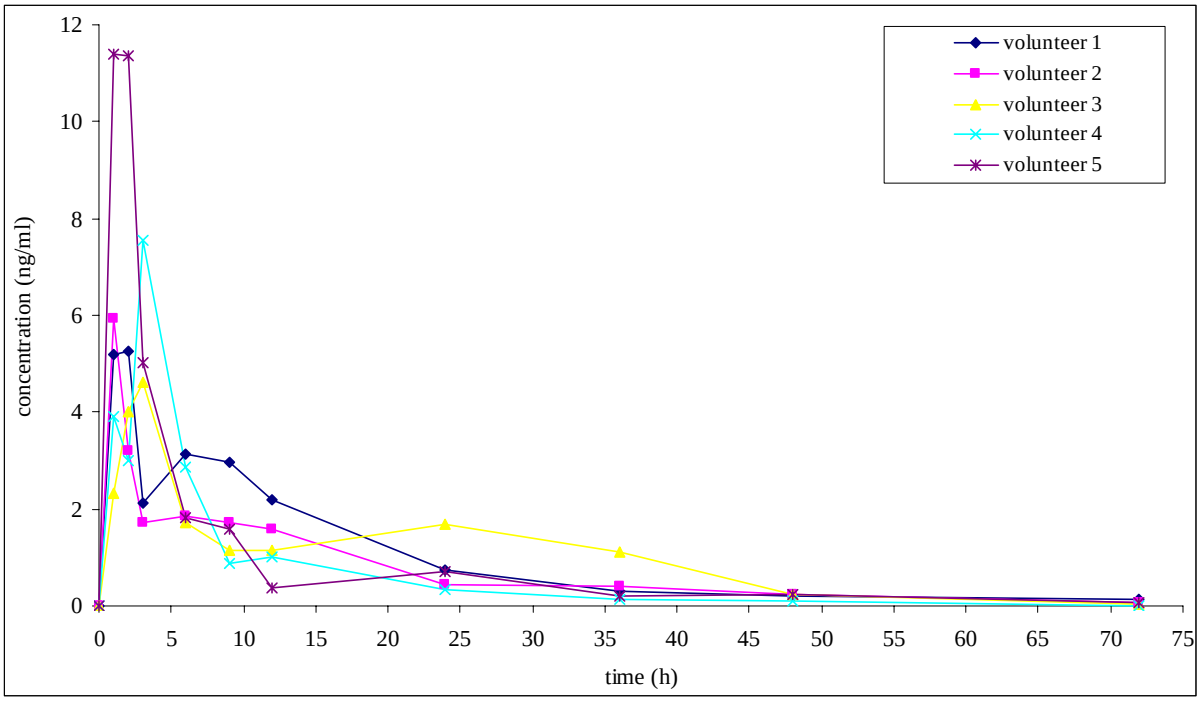

Figure 2: Concentrationprofiles of excreted formoterol.

Detection times ranged up to 72 hours. Cumulative excretion data showed that the total amount of unchanged drug excreted during the first 12 hours varied between 2.5 and 4.3 µg corresponding to 14 and 23 % of the administered dose.

Terbutaline

For all volunteers similar excretion curves were observed and terbutaline could be detected already 1 hour after intake (Figure 3). The maximum urinary concentration was reached between 1 and 3 hour for all volunteers. The peak concentrations ranged between 54 and 178 ng/ml.

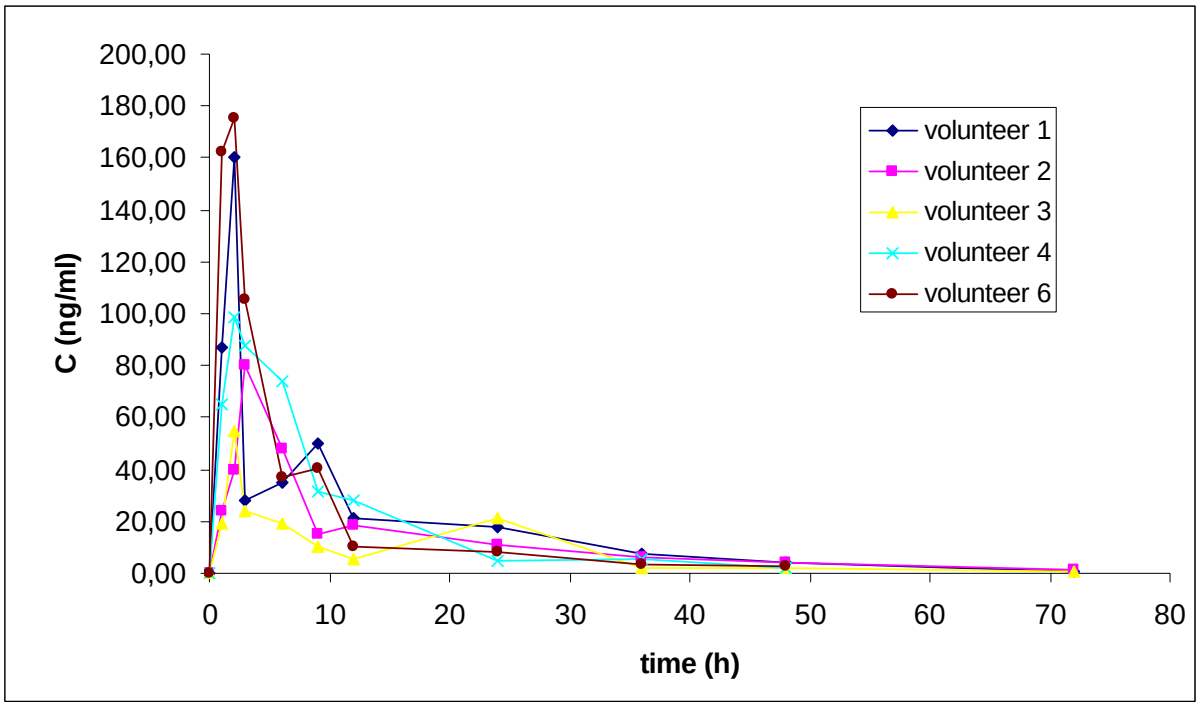

Figure 3: Concentrationprofiles of excreted terbutaline.

Due to the high concentrations, terbutaline could be detected up to the timepoint where the collection of the urine was stopped, i.e. 72 hours.

Cumulative excretion data showed that the total amount of unchanged drug excreted during the first 12 hours varied between 8.5 and 17 % of the administered dose.

Conclusions

Generally, great differences were observed in urinary concentrations for all investigated preparations. These can be attributed to differences in urinary volumes produced as well as the pH of the urine, which can influence the excretion of the basic β_2-agonists.
Additionally, for all preparations could be concluded that maximum 23% of the administered dose could be recovered in the urine as parent drug. This can be partially attributed to the poor use of the inhalation device [17].
The results of this study also shows that only for terbutaline the minimum required reporting level (MRPL) of 100 ng/ml can be exceeded after normal therapeutic use. Salmeterol and formoterol were excreted in much lower concentrations than the MRPL. Hence to control their use it is recommended to lower the MRPL of this group of substances.
The main goal of this study was to determine which concentrations can be detected in urine after therapeutical application. The results presented in this study can help national anti-doping organizstions to assign the detected concentrations in the routine samples to therapeutic use or to doping misuse.

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