Quantitative detection of inhaled formoterol in human urine and relevance to doping control analysis

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Short title: Detection of formoterol in urine.

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Abstract

Formoterol is a frequently prescribed β_2-agonist used for the treatment of asthma. Due to performance enhancing effects of some β_2-agonists, formoterol appears on the prohibited list, published by the World Anti-doping Agency (WADA). Its therapeutic use is allowed but restricted to inhalation. Since the data on urinary concentrations originating from therapeutic use is limited, no discrimination can be made between use and misuse when a routine sample is found to contain formoterol. Therefore the urinary excretion of 6 volunteers after inhalation of 18 µg of formoterol was investigated.

An LC-MS/MS method was developed and validated for the quantification of formoterol in urine samples. Sample preparation consists of an enzymatic hydrolysis of the urine samples, followed by a liquid-liquid extraction at pH 9.5 with diethyl ether/isopropanol (5/1, v/v). Analysis was performed using selected reaction monitoring after electrospray ionisation. The method was linear in the range of 0.5-50 ng/mL. The limit of quantification (LOQ) was 0.5 ng/mL. The bias ranged between -1.0 and -6.8 %. Results for the urinary excretion show that formoterol could be detected for 72 hours. The maximum urinary concentration detected was 8.5 ng/mL without and 11.4 ng/mL after enzymatic hydrolysis. Cumulative data showed that maximum 11.5 % and 23 % of the administered dose is excreted as parent drug within the first 12 hours, respectively non-conjugated and conjugated.

Analysis of 82 routine doping samples, declared positive for formoterol during routine analysis, did not exhibit concentrations which could be attributed to misuse.

Introduction

Formoterol is a potent long-acting β_2-adrenergic agonist and has a pronounced and very effective bronchodilating effect [1]. Consequently, it is amongst the most prescribed drugs for humans in the treatment of asthma. Besides the desired pharmacological action ,some β_2-agonists produce side-effects on protein synthesis and lipolysis resulting in anabolic action at higher doses [2]. Hence β_2-agonists might be misused in sports for the stimulatory effects on the respiratory and central nervous system and for growth-promoting action. To control the use of β_2-agonists, the World Anti Doping Agency (WADA) included them in the list of prohibited substances [3] and imposed a minimum required performance level (MRPL) of 100 ng/mL [4].

Before the introduction of liquid-chromatography mass spectrometry (LC-MS), the detection of β_2-agonists in the field of doping analysis was performed by gas chromatography-mass spectrometry (GC-MS) [5]. For formoterol N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) in combination with trimethylsilyl-imidazole (TMSIm) is the preferred choice to derivatize all groups including the nitrogen of the β-ethanolamine chain [6]. Monitoring formoterol in our laboratory by GC-MS after basic liquid-liquid extraction and derivatisation never resulted in an adverse analytical finding (AAF) for this substance. Liquid chromatography-mass spectrometry (LC-MS) has proven to be an effective tool in the urinary detection of β_2-agonists related to doping control analysis [7-10]. Its application for β_2-agonist-detection in our laboratory since 2007, resulted in numerous urine samples which were found to contain formoterol (non published results).

Because of therapeutic importance, the use of formoterol is widespread and the current (2011) situation is that the athlete should have a therapeutic use exemption (TUE). Consequently, formoterol is frequently declared on doping control forms [11]. Because the information on urinary concentrations of formoterol after inhalation is limited to one paper describing an administration study with 2 volunteers [5], it is difficult to assign the detected concentrations in the routine samples to therapeutic use or to doping misuse. Therefore, the objective of this study was to investigate the urinary excretion of a therapeutic dose of formoterol after inhalation. Additionally, concentrations from the excretion study will be compared with those observed in routine samples.

Experimental

Products and reagents

Formoterol was obtained from Novartis (Ringaskiddy, Ireland) and formoterol-d_6 (internal standard) (IS) from Medical-Isotopes (Pelham, USA). The preparation Oxis (formoterol fumarate) was from Astra Zeneca (Brussels).

Acetic acid (HOAc) p.a., sodium acetate (NaOAc) p.a., isopropanol, diethyl ether, dipotassium carbonate (K_2CO_3), sodium hydrogen carbonate ($NaHCO_3$) and sodium hydroxide (NaOH) were of analytical grade and were purchased from Merck (Darmstadt, Germany). Methanol (MeOH), ammonium acetate (NH_4OAc) and HPLC grade water were from Biosolve (Valkenswaard, The Netherlands). Beta-glucuronidase containing 145700 units/mL glucuronidase and 714 units/mL aryl-sulphatas from *Helix Pomatia* was from Sigma-Aldrich (Bornem, Belgium). The buffer (pH 5.2) was obtained by dissolving 136 g NaOAc into 800 mL of aqua bidest. The pH was adjusted, if necessary, to 5.2 by adding HOAc. Then the final volume was made to 1L. Buffer (pH 9.5) was prepared by dissolving 45 g K_2CO_3 and 37 g $NaHCO_3$ in 300 mL of H_2O.

Instrumentation

The HPLC system consisted of a Surveyor MS-pump and Surveyor autosampler with a 50 µL sample loop (Thermo, San Jose, CA, USA). Twenty microliter of sample was injected. Separation was performed on a Zorbax RX C8-column (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_4OAc and 0,1% HOAc. Gradient elution at a flow rate of 0.4 mL/min was performed as follows: 65% A for 0.5 min decreased to 20% A in 11.5 min and an increase to the initial condition of 65% A in 0.1 min followed by an equilibration step of 2.4 min before the next injection. Total analysis time per sample was 14.5 min. The LC effluent was pumped to a Quantum Discovery mass spectrometer (Thermo) 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 units. No auxiliary gas was used. The mass spectrometer was operated in selected reaction monitoring (SRM) mode and transitions are presented in table 1. The precursor ions were selected in the first quadrupole with a peak width at half maximum (FWHM) of 0.7.

The scan speed and scan width were maintained at 100 ms and 0.01 amu, respectively. The collision gas pressure was 1.5 mTorr.

Sample preparation

The internal standard (IS)-solution (50 µL, 100 ng/mL of formoterol-d_6 in MeOH) was added to 1 mL of urine, followed by the addition of 1 mL of acetate buffer (pH 5.2) and 50 µl of the enzyme solution. 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 diethyl ether/ isopropanol (5/1). After centrifugation at 1.5 G the organic layer was transferred into a new tube and evaporated until dry at 40°C under oxygen free nitrogen (OFN). The residue was dissolved in 200 µL of the initial mobile phase composition.
For the analysis without hydrolysis the addition of the acetate buffer, and the enzyme solution as well as the incubation were omitted.

Method validation

A six-point calibration curve was generated by spiking blank urine with methanolic formoterol solutions in triplicate at 0.5, 1, 5, 10, 25 and 50 ng/mL. The ratio's of target compound product ion area to IS product ion were plotted versus concentration to obtain calibration curves. Precision and bias were tested at the lowest, middle and highest calibrator. Precision was assessed as the percentage relative standard deviation (%RSD) of both repeatability (within-day) (n=6) and reproducibility (between-day and different analysts) (n=18) for a selected level. Maximum allowed tolerances for precision 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 [12]. Bias was defined as the difference between the calculated mean amount and the specified amount as a percentage [13]. The limit of quantification (LOQ) of the method was defined as the lowest concentration where precision and bias were within the above mentioned criteria. 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 urine samples as described above 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 formoterol and IS-solution. After evaporating the organic solvent, the 6 tubes, containing the extracted matrix and an additional tube (=reference) were spiked with formoterol at 10 ng/mL and with 50 µL of the internal standard solution. After evaporating the methanolic solutions, the remaining residues were dissolved in 200 µL of the initial mobile phase and analysed. Then, the ion suppression was determined by comparing the peak areas for formoterol in the extracted urine samples with the peak area for formoterol in the reference sample. To evaluate the corrective effect of the IS on the ion suppression, area ratio's of formoterol and the IS were compared with the area ratio of formoterol and the IS in the reference sample.

Excretion study

The study was performed with 6 healthy male 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 and inhaled 18 µg formoterol (2 puffs of 9 µg) using an Oxis Turbohaler. Urine samples were collected before (0 h) 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 pH were measured and all samples were analysed in duplicate.

Collection of routine samples

During a one year period doping control samples in which formoterol was detected during routine doping analysis were collected and stored at -20°C awaiting analysis.

Results and discussion

Method development

As already stated in the introduction LC-MS is the preferred detection technique for β_2−agonists. The excellent LC-MS sensitivity of formoterol can be attributed to the presence of the basic nitrogen which is easily protonable during the electrospray ionisation, resulting in abundant precursor ions. MS/MS fragmentation of formoterol results in specific product ions which were explained by Thevis et al. [7]. Due to the amphoteric character of β_2-agonists (phenolic hydroxyls and amine function) the optimal extraction pH can differ for this class of compounds [2]. Henze et al. investigated the extraction behaviour of β_2-agonists thoroughly [14]. Formoterol showed a quasi constant extraction behaviour between pH 5 and 11. Because extraction buffers between pH 9-10 are routinely used in doping control laboratories and have proven to result in clean extracts for the determination of β_2-agonists [5], a buffer at pH 9.5 was preferred.
According to the literature, β_2-agonists can be both excreted free, glucuronidated and sulphated [2,15]. Therefore β-glucuronidase containing also aryl-sulphatase activity was selected to hydrolyse the samples. Nevertheless, formoterol is predominantly excreted in urine conjugated as glucuronides [16-18]. According to Rosenborg et al. [17] only 4.8% of an administered dose of formoterol and deformylated formoterol are excreted in urine as 4'OH-sulphate metabolites.

Method validation

Using a least square fit, good linearity ($r^2 \geq 0.98$) was observed. The calibration curve was not forced through the origin and for the regression calculation a weighing factor of 1/x was used for all data points.
The results for precision and bias are summarised in Table 2 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 (bias) was below the acceptable threshold of 15% and 20 % for all levels in the range of the calibration curve [13]. The limit of quantification (LOQ) of the method was 0.5 ng/mL.
Regarding the selectivity, interferences from other monitored 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.

Determining the ion suppression showed an average value of 30 % with an RSD of 19 % across the six urine samples. This high average value can be explained by the relatively large amount of matrix extracted by the diethyl ether/isopropanol mixture.

The RSD of 19 % indicates a high variation of the ion suppression depending on the individual samples. This observation requires the correction by an adequate internal standard in order to obtain correct quantification. After correcting the areas of formoterol with the areas of the deuterated IS, the effect of the ion suppression was reduced to -3.4 % with an RSD of 3.5 %.

Application to excretion urine samples

For all volunteers formoterol could be detected already 1 hour after intake (Figure 1). The maximum urinary concentration was reached between 1 and 3 hours. The peak concentrations ranged between 1 ng/mL and 8.3 ng/mL without hydrolysis (Figure 2) and between 2.3 and 11.4 ng/mL with hydrolysis (Figure 3). The observed concentrations in this study are in agreement with the concentrations observed by Ventura et al. [5] which describe maximum urinary concentrations of 8.5 and 17.5 ng/mL for two volunteers, respectively, after inhalation of 24 µg formoterol.

With hydrolysis, detection times reached up to 72 hours for some volunteers whereas without hydrolysis step the detection time was limited to 36 hours for all volunteers.

The cumulative excretion profiles are presented in figures 3 and 4. The total amount of unchanged drug excreted during the first 12 hours varied between 1.1 and 2.0 µg without hydrolysis (Figure 4) and 2.5 and 4.3 µg with hydrolysis (Figure 5) corresponding to 6.3 to 11.5 % and 14 to 23 % of the administered dose, respectively. These results are in agreement with a previous paper describing that 8 % of an inhaled dose is recovered in urine as free formoterol [19] and 35% for the total fraction (both free and glucuronides)[17].

Also the difference observed in the amounts excreted free and conjugated is in accordance with previous work, which describe that formoterol is predominantly excreted conjugated more specific as glucuronides [16-18]. Large individual differences were found in the urinary concentrations as well as the excreted amounts. This variation can be assigned partially to the urinary pH and the urinary flow, which can influence the excretion of basic compounds [20].

However, it can not be excluded that the variations are also caused by a poor use of the inhalation device [21].

Application to routine samples

During a one year period routine samples, in which formoterol was detected, were collected to determine the urinary concentrations and compare these with the ones obtained from the excretion study. A total of 7045 samples were screened and 82 samples were found to contain formoterol (1.1 %). Nearly twice as many samples containing formoterol were detected compared to a previous study where salmeterol was monitored [8]. Indeed, formoterol is the most popular β_2-agonist used for the treatment of exercise induced asthma.
The histogram showing the distribution of the detected concentrations is presented in figure 5. The highest detected concentration was 20.8 ng/mL whereas in the excretion study the maximum observed concentration was 11.4 ng/mL (Figure 6).
The concentrations obtained in our study are obtained after a normal day dose of 2 inhalations. Taking into account that the dose can be increased to 6 inhalations (= total daydose of 54 µg per day) in severe cases of asthma [22], the sample in which 20.8 ng/mL was detected can be the result of such a situation.
Besides, if misuse of formoterol would be widespread or higher therapeutic doses would be used much more routine samples would show concentrations higher than those obtained during the excretion study.

Conclusion

A sensitive LC-ESI/MS/MS method for the quantification of formoterol in urine was developed and validated. The method was successfully applied to urine samples from an administration study and to urine samples collected during routine analysis. The results of the excretion study show that after inhalation of 18 µg formoterol, the parent substance could be detected up to 72 hours. The peak concentrations in urine were between 2.3 and 11.4 ng/mL. Excreted amounts show that inhaled formoterol is predominantly excreted conjugated. Taking into account the in

this study observed maximum concentration, the current WADA MRPL of 100 ng/mL is too high to detect inhaled formoterol after therapeutical application. Comparison of the urinary concentrations obtained during the excretion studies with the concentrations in routine doping samples did not allow to conclude that formoterol is misused by athletes for its performance enhancing effects.

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Legends to figures:

Figure 1: Extracted ion chromatograms for a blank sample before inhalation (b), urine sample 1h after inhalation of 18 ùg formoterol (c). transitions 345=> 121 and 149 are for formoterol, transition 351 => 155 are for formoterol-d_6.

Figure 2: Concentration profiles of excreted formoterol analysed without hydrolysis

Figure 3: Concentration profiles of excreted formoterol analysed with hydrolysis

Figure 4: Cumulative excretion curves of formoterol (0-12h) analysed without hydrolysis

Figure 5: Cumulative excretion curves of formoterol (0-12h) analysed with hydrolysis

Figure 6: Histogram showing distribution of detected formoterol concentrations in 82 routine samples.

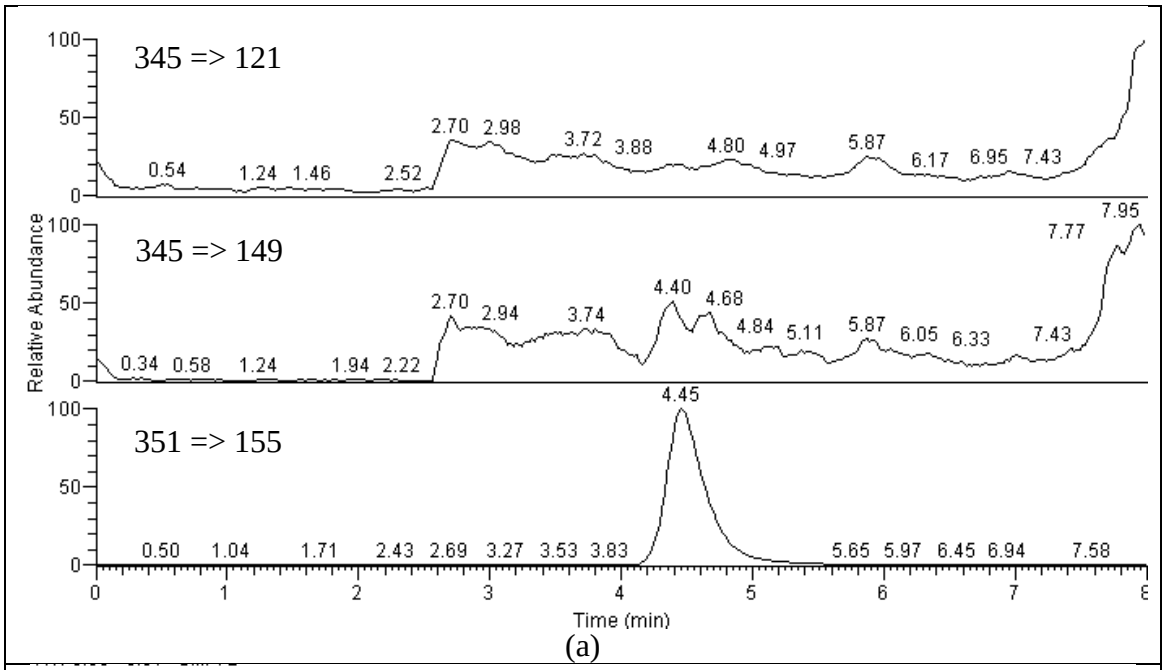
Table 1: MS/MS detection settings for formoterol and formoterol-d_6

$[M+H]^+$	CE	DI	TLV
345	35	93	123
	54	106	123
	34	121	123
	36	134	123
	19	149*	123
351	19	155	123

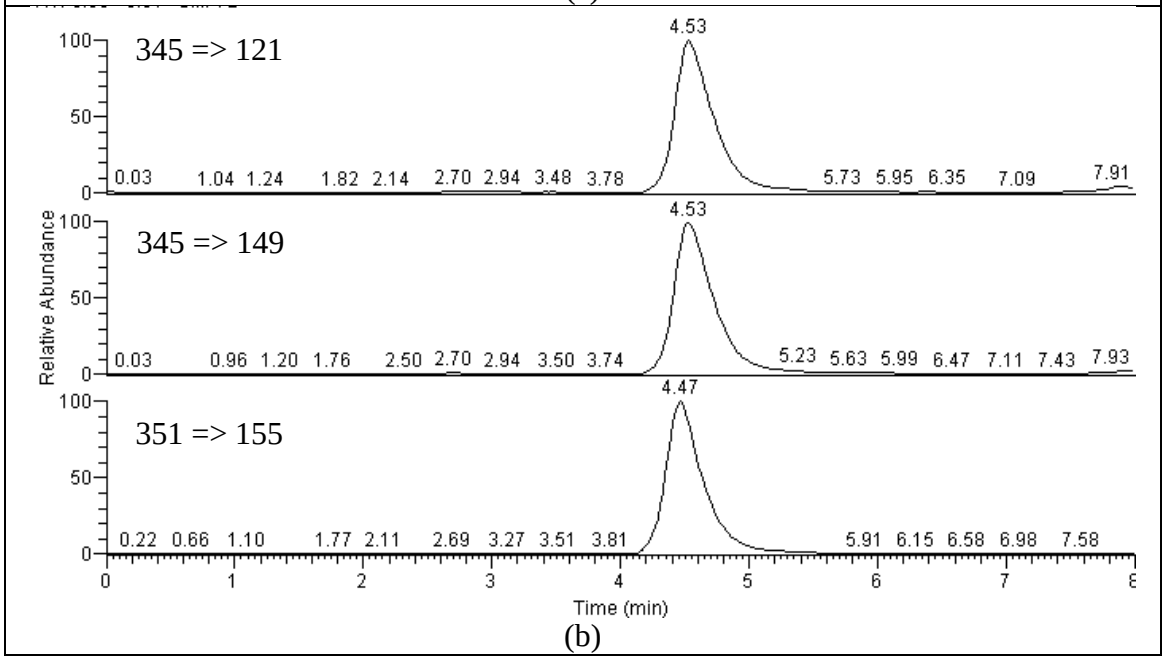
CE: collision energy, DI: diagnostic ion, TLV : Tube Lens Voltage, *quantifier ion

Table 2: Bias, repeatability, reproducibility and tolerance limits of the LC-MS/MS method including the lowest and highest point of the calibration curves.

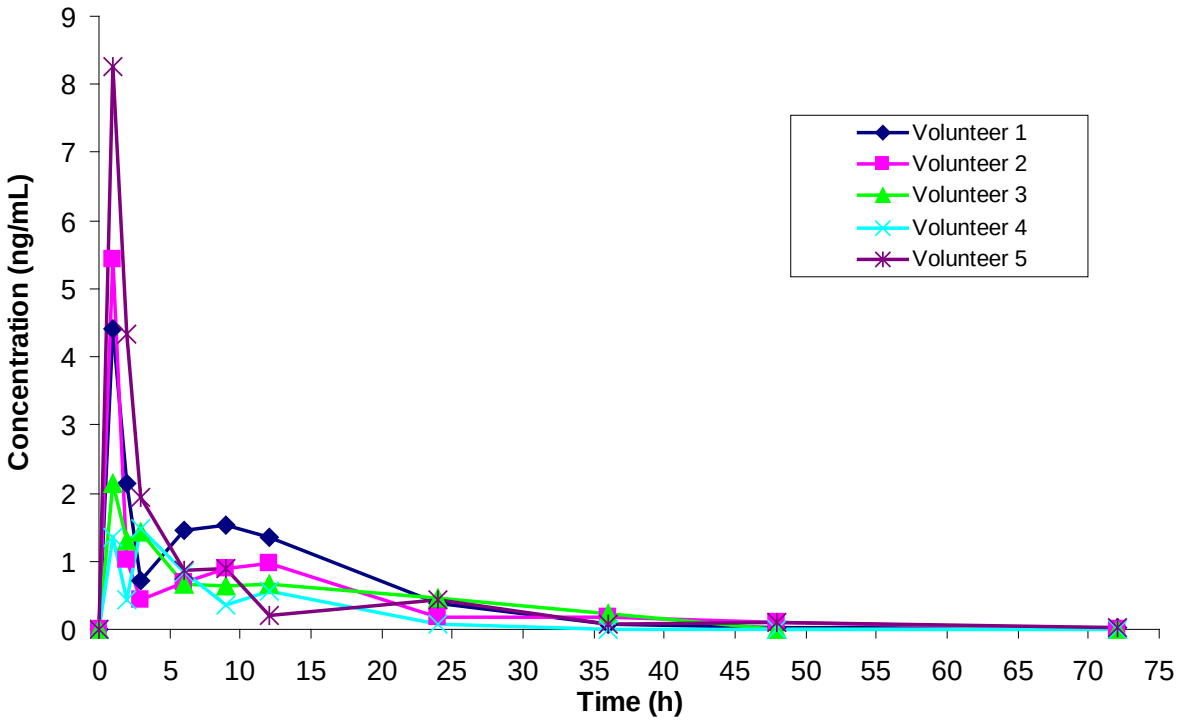
Conc (ng/mL)	Repeatability Bias (%)	Reproducability Bias (%)	Repeatability RSD (%)	Reproducibility RSD (%)	RSDmax (%)	2/3RSDmax (%)
0.5	-1.0	-6.8	17.1	13.3	48	32
10	-0.8	-2.9	1.8	2.0	32	21
50	-0.05	-0.3	1.6	1.0	25	16


345 => 121
345 => 149
351 => 155
Relative Abundance
Time (min)

(a)


345 => 121
345 => 149
351 => 155
Relative Abundance
Time (min)

(b)


Concentration (ng/mL)
Time (h)
Volunteer 1
Volunteer 2
Volunteer 3
Volunteer 4
Volunteer 5
0
1
2
3
4
5
6
7
8
9
0
5
10
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25
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45
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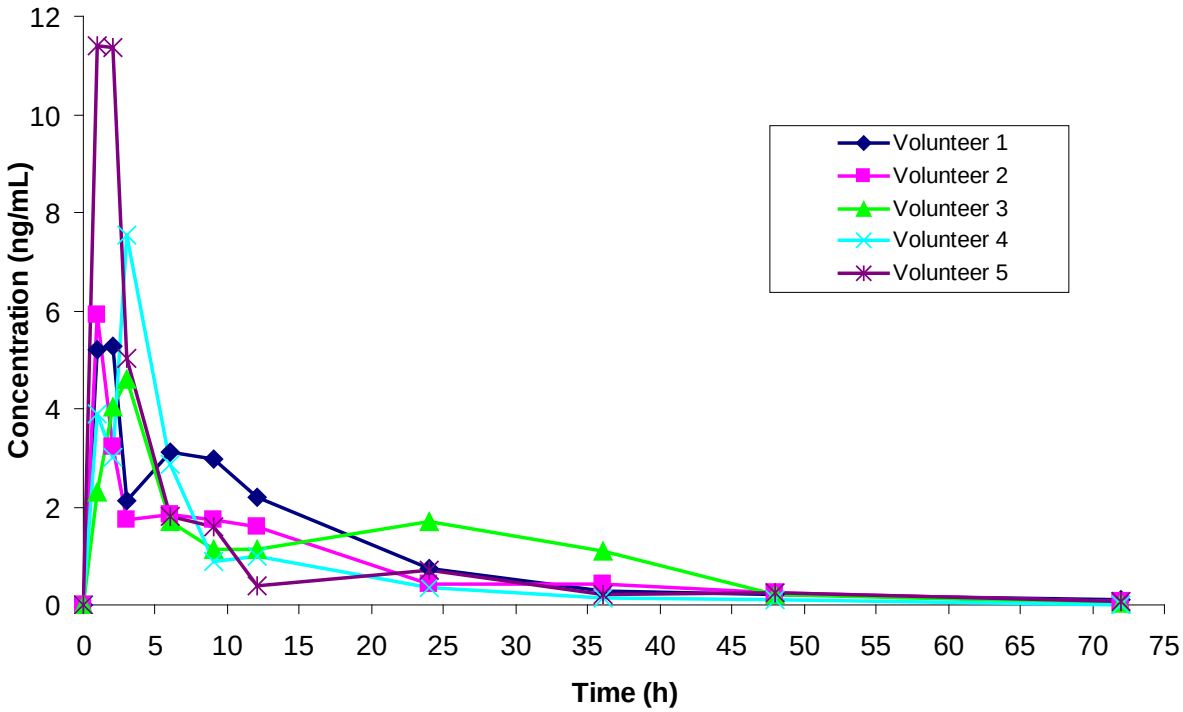
65
70
75


Concentration (ng/mL)
Time (h)
Volunteer 1
Volunteer 2
Volunteer 3
Volunteer 4
Volunteer 5
0
2
4
6
8
10
12
0
5
10
15
20
25
30
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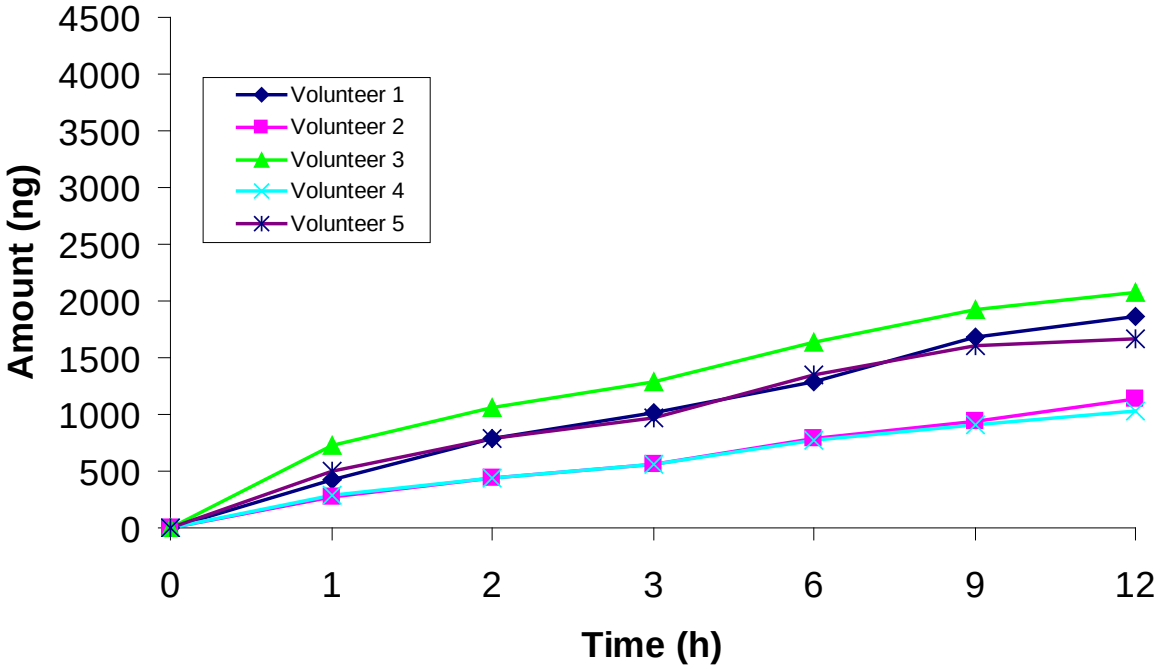
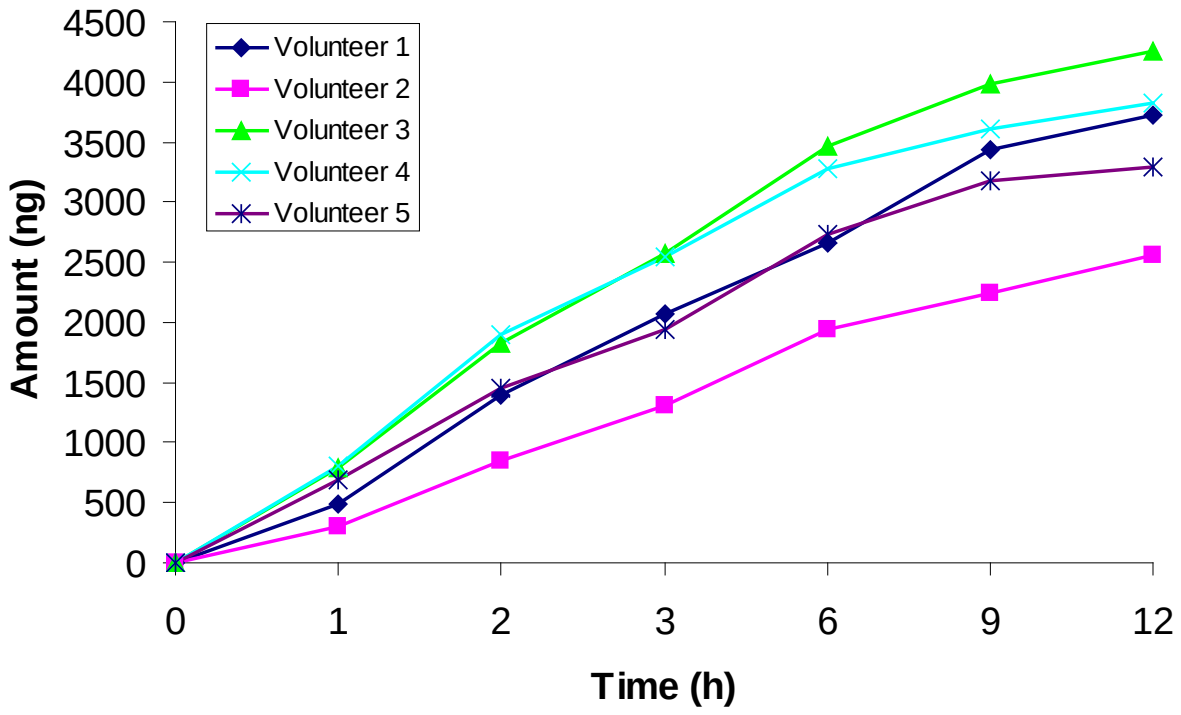
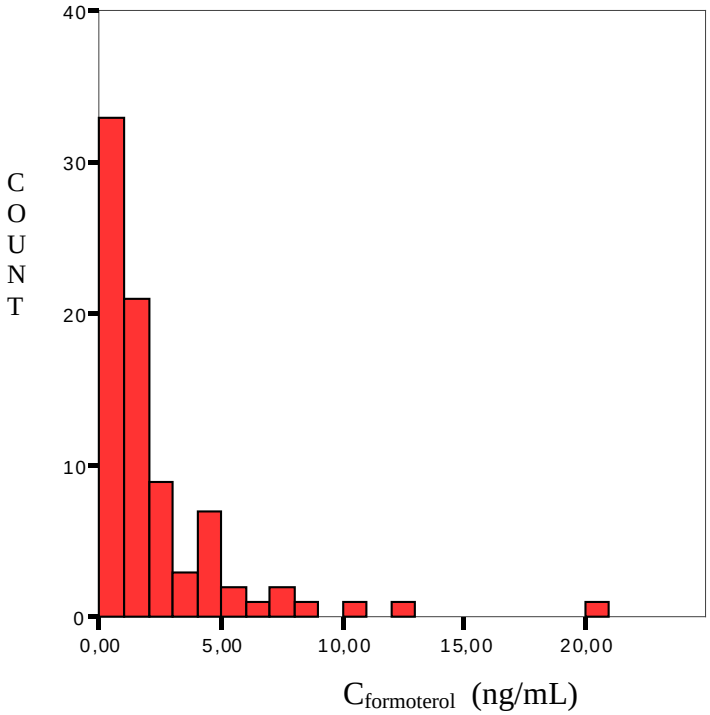
40
45
50
55
60
65
70
75


4500
4000
3500
3000
2500
2000
1500
1000
500
0
Amount (ng)
Volunteer 1
Volunteer 2
Volunteer 3
Volunteer 4
Volunteer 5
0
1
2
3
6
9
12
Time (h)


4500
4000
3500
3000
2500
2000
1500
1000
500
0
Amount (ng)
Volunteer 1
Volunteer 2
Volunteer 3
Volunteer 4
Volunteer 5
0
1
2
3
6
9
12
Time (h)


40
30
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10
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COUNT
0,00
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10,00
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20,00
Cformoterol (ng/mL)