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Determination of salbutamol and salbutamol glucuronide in human urine by means of liquid chromatography-tandem mass spectrometry

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ABSTRACT: The determination of salbutamol and its glucuronide in human urine following the inhalative and oral administration of therapeutic doses of salbutamol preparations was performed by means of direct urine injection utilizing liquid chromatography-tandem mass spectrometry (LC-MS/MS) and employing d_3-salbutamol and d_3-salbutamol glucuronide as internal standards. Unconjugated salbutamol was detected in all administration study urine samples. Salbutamol concentrations following inhalation were commonly (99%) below 1000 ng/ml whereas values after oral administration frequently (48%) exceeded this threshold.

While salbutamol glucuronide was not detected in urine samples collected after inhalation of the drug, 26 out of 82 specimens obtained after oral application contained salbutamol glucuronide with a peak value of 63 ng/ml. The percentage of salbutamol glucuronide compared to unconjugated salbutamol was less than 3%.

Authentic doping control urine samples indicating screening results for salbutamol less than 1000 ng/ml, showed salbutamol glucuronide concentrations between 2 and 6 ng/ml, whereas adverse analytical findings resulting from salbutamol levels higher than 1000 ng/ml, had salbutamol glucuronide values between 8 and 15 ng/ml.

The approach enabled the rapid determination of salbutamol and its glucuronic acid conjugate in human urine and represents an alternative to existing procedures since time-consuming hydrolysis or derivatization steps were omitted.

Moreover, the excretion of salbutamol glucuronide in human urine following the administration of salbutamol was proven.

Keywords: salbutamol; salbutamol glucuronide; doping; urine; sport

Introduction

According to World Anti-Doping Agency (WADA) regulations[1] the presence of salbutamol in urine in excess of 1000 ng/ml is presumed to be incompatible with a therapeutic use of the substance and will be considered as an Adverse Analytical Finding (AAF) unless the athlete proves, through a controlled pharmacokinetic study, that the abnormal result was the consequence of the use of a therapeutic dose (maximum 1600 micrograms over 24 h) of inhaled salbutamol.

The threshold concentration is based on the sum of the glucuronide conjugate (expressed as the free drug) and free drug concentrations.[2]

Numerous articles on the analysis of salbutamol in urine have been published over the last decade and extensive investigations concerning the discrimination of the prohibited oral use of salbutamol in sport from authorized inhaled asthma treatment[3–6] using conventional doping control screening procedures led to a threshold value of 1 μg/ml. The identification and quantification of salbutamol in urine has commonly been accomplished by means of gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) following solid phase extraction (SPE) sample preparation.[7–9] Being employed in experiments concerning the stability of salbutamol in native urinary matrix, the analytical methods outlined a decrease of the target compound of as much as 80–90% within 25 days after storage at +4 °C / −18 °C.[10,11]

Concentrations of the glucuronide conjugate and free drug showed considerable variations in excretion study urine samples;[12–14] hence, the established threshold was based on the sum of the glucuronide conjugate and the free drug concentration.

Screening for salbutamol is commonly performed by extraction of urine at pH 9.6 with *tert*-butyl methyl ether after enzymatic hydrolysis with *E.coli*, derivatization with MSTFA/NH_4I/ethanethiol and GC/MS analysis.[15–17] In-house confirmatory analysis was also based on liquid-liquid extraction after enzymatic hydrolysis following liquid chromatography-tandem mass spectrometry (LC-MS/MS) using deuterated salbutamol as internal standard (unpublished results).

Because of the time-consuming sample preparation of salbutamol in urinary matrix, an alternative method for the analysis of salbutamol in doping control samples was needed, and the option of direct injection of urine after addition of stable isotope-labelled salbutamol was evaluated in accordance with earlier studies.[18–21]

Based on this methodology, an alternative approach for the quantitative determination of salbutamol in doping control samples was

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developed in 2007 utilizing LC-MS/MS.[22] The urine samples were fortified with the internal standard deuterated **d_6**-salbutamol (500 ng/ml) and, without any further sample preparation, aliquots of 10 µl were injected into the LC-MS/MS instrument allowing a lower limit of detection (LOD) of 20 ng/ml. Excretion study urine samples following oral or inhalative administration of salbutamol preparations were analyzed with regard to salbutamol and its conjugates, and the administered drug as well as the corresponding sulfate were observed while no glucuronide was detectable.

In order to corroborate or complement these findings, the objective of the present study was to probe for salbutamol and salbutamol glucuronide in urine samples collected following the inhalative and oral administration of therapeutic doses of salbutamol preparations.

The question whether salbutamol glucuronide is detectable in human urine after administration of salbutamol is of considerable interest for the selection of the analytical assay, since the time-consuming hydrolysis step could be omitted if only negligible amounts of salbutamol are produced and renally excreted in humans.

Experimental

Chemicals and reagents

All solvents and reagents were of analytical grade purity. Salbutamol was obtained from Riedel-de Haën (Seelze, Germany). The internal standard **d_3**-salbutamol was purchased from CDN Isotopes (Quebec, Canada). NADPH was purchased from Roche Diagnostics (Mannheim, Germany) and uridine 5´-diphosphoglucuronic acid (UDPGA) from Sigma (St Louis, MA, USA). Rat liver microsomes were obtained from BD Gentest (Woburn, MA, USA). All solutions and buffers were prepared using deionized water (Water Lab System, Millipore, Eschborn, Germany).

Salbutamol

Sample preparation for the direct quantification of salbutamol

Urine samples (1 ml) were fortified with 500 ng of the internal standard **d_3**-salbutamol and transferred into HPLC vials. A volume of 10 µl was injected into the LC-MS/MS instrument.

Salbutamol glucuronide and d_3-salbutamol glucuronide

*In vitro synthesis of salbutamol glucuronide and **d_3**-salbutamol glucuronide*

The *in vitro* synthesis of salbutamol glucuronide and the internal standard **d_3**-salbutamol glucuronide was performed according to a modified assay from Kuuranne *et al*.[23,24] Pooled rat liver microsomes of male Wistar rats were used for the enzymatic reaction, since the major urinary metabolite of salbutamol in rats (rabbits and dogs) is known to be salbutamol 4´-O-glucuronide.[25] In contrast, human liver microsomes were not considered resulting from the fact that salbutamol is excreted in human urine mainly unconjugated or conjugated as 4´-O-sulfate.[26]

In brief, salbutamol stock solution (24 µg/ml) was evaporated to dryness and the residue dissolved in 50 mM phosphate buffer (pH 7.4) containing 5 mM $MgCl_2$ at substrate concentration 100 µM and 5 mM NADPH. The reaction was initiated by the addition of UDPGA to a final concentration of 10 mM and carried out at 37 °C for 3 h. The reaction was terminated by the addition of 200 µl acetone and the reaction mixture was then centrifuged (17 000 x *g*, 10 min). The organic layer was evaporated from the supernatants *in vacuo* before further sample preparation. The dry residue was dissolved in 50 µl H_2O and purified via SPE extraction.

For the *in vitro* synthesis of **d_3**-salbutamol glucuronide 20µL of a 5 mM stock solution was utilized and treated as mentioned above.

*Quantitative analysis of the salbutamol glucuronide and **d_3**-salbutamol glucuronide reference material*

The determination of the salbutamol concentration in the synthesized reference material was performed by means of LC-MS/MS following enzymatic hydrolysis at pH 7 with β-glucuronidase from *E.coli* utilizing a calibration curve.

Sample preparation for the determination of salbutamol glucuronide

Two ml of urine and 20 ng of the internal standard **d_3**-salbutamol glucuronide were applied on a freshly prepared and conditioned solid phase extraction column (PAD-1 adsorber resin), washed with deionized water and eluted with 2 ml of methanol. The eluate was evaporated to dryness, reconstituted in 100 µL of MeOH / ammonium acetate buffer (20:80) and a volume of 10 µl was injected into the LC-MS/MS instrument.

Analysis for salbutamol glucuronide

The screening analysis for salbutamol glucuronide was performed in all samples by means of an Agilent 1200 series liquid chromatograph coupled to an Applied Biosystems API 4000 Qtrap with electrospray ionization (ESI). Specimens showing suspicious screening results were then re-analyzed using a more sensitive instrumental setup (Agilent 1100 LC; Applied Biosystems API 5500 Qtrap (ESI)).

Assay validation for salbutamol and salbutamol glucuronide

The assay validation for salbutamol was initially performed in 2007,[22] utilizing **d_6**-salbutamol as internal standard. Due to the fact, that the internal standard was changed to **d_3**-salbutamol, a cross validation was conducted for the direct quantification of salbutamol.

The reason for the alteration of the internal standard is the arrangement of the deuterium atoms in the respective chemical structure. While **d_6**-salbutamol bears all deuterium labels in the *tert*-butyl residue, the deuterium atoms of **d_3**-salbutamol are located at carbons X and Y (Figure 1b), thus leading to different and more favorable dissociation patterns and product ion abundances.

Specificity

Six different blank urine specimens obtained from female and male healthy volunteers were prepared and analyzed as described above in order to probe for interfering peaks in the selected ion chromatograms at the expected retention time of the analytes.

Lower limit of detection (LLOD) and lower limit of quantification (LLOQ)

The LLOD was defined as the 'lowest content that can be measured with reasonable statistical certainty' at a signal-to-noise ratio ≥ 3, while the LLOQ represents the lowest quantifiable amount of an analyte, which is characterized by a signal-to-noise ratio ≥ 9.[27] Six blank urine samples spiked with the internal standard (ISTD) only, and six urine specimens fortified with the analyte (salbutamol: 5 ng/ml; salbutamol glucuronide: 2 ng/ml) were prepared and analyzed according to the established protocol providing the data necessary to estimate the LLOD and LLOQ.

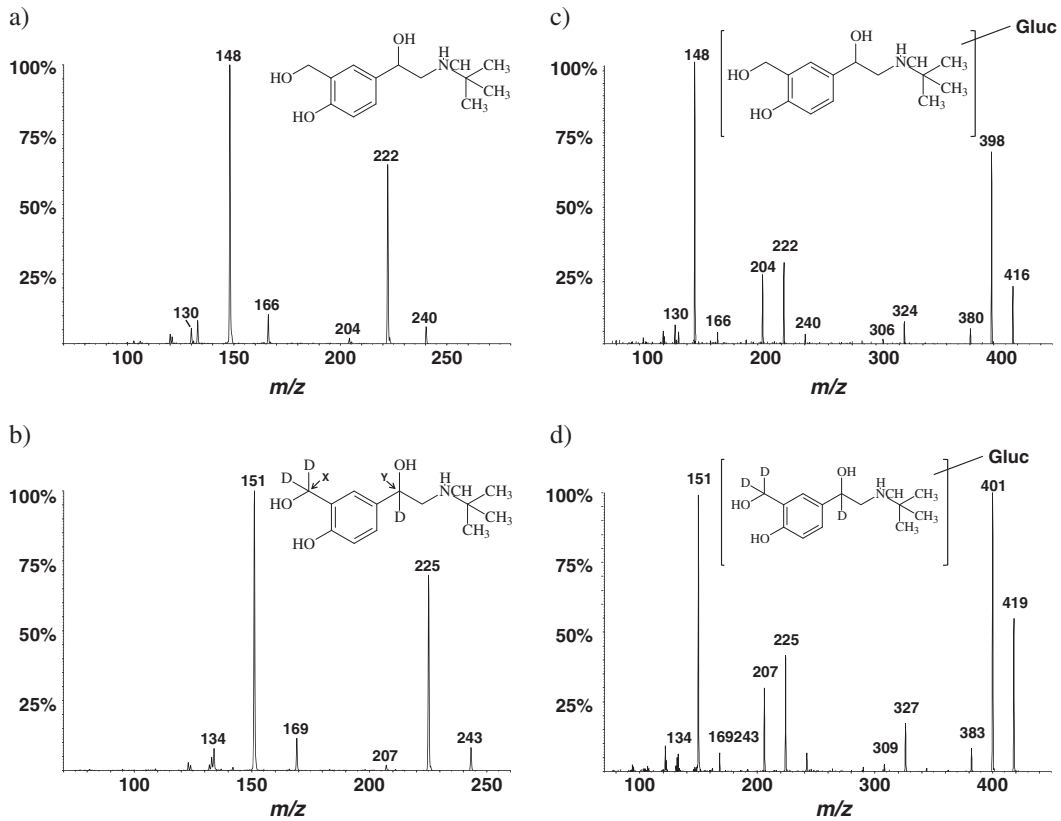

Figure 1. Product ion mass spectra, recorded on an Applied Biosystems API 4000 Qtrap mass spectrometer using a CE of 30 eV of (a) salbutamol ([M + H^+] = 240); (b) **d_3**-salbutamol ([M + H^+] = 243); (c) salbutamol glucuronide ([M + H^+] = 416); and (d) **d_3**-salbutamol glucuronide ([M + H^+] = 419).

Linearity

Calibration curves for salbutamol and salbutamol glucuronide were generated using blank urine spiked at 250 – 500 – 750 – 1000 – 1250 – 1500 – 1750 – 2000–2250 and 2500 ng/ml (salbutamol), respectively 5 – 10 – 20 – 30 – 50 and 60 ng/ml (salbutamol glucuronide). The peak area ratios of analyte and ISTD were utilized to calculate the correlation coefficient, intercept, and slope.

Intra-day precision

On one day, six urine samples of low (salbutamol: 500 ng/ml; salbutamol glucuronide: 5 ng/ml), medium (salbutamol: 1000 ng/ml; salbutamol glucuronide: 20 ng/ml) and high (salbutamol: 2000 ng/ml; salbutamol glucuronide: 40 ng/ml) concentrations were prepared, analyzed randomly, and the assay precision was calculated for each concentration level.

Inter-day precision

On three consecutive days, six urine samples of low (salbutamol: 500 ng/ml; salbutamol glucuronide: 5 ng/ml), medium (salbutamol: 1000 ng/ml; salbutamol glucuronide: 20 ng/ml) and high (salbutamol: 2000 ng/ml; salbutamol glucuronide: 40 ng/ml) concentrations were prepared, analyzed randomly, and the assay precision was calculated for each concentration level.

Recovery

The recovery for salbutamol glucuronide was determined from urine aliquots, which were prepared and analyzed for the inter-day precision. The results obtained were correlated with the calibration curve.

Extraction efficiency

The extraction efficiency for salbutamol glucuronide was calculated by comparison of the peak area ratios from six commonly fortified urine samples (at 20 ng/ml) and six urine samples that were fortified with the same amount after sample preparation prior to evaporation of the solvent.

Ion suppression

Ion-suppression studies were performed using post-column continuous infusion of salbutamol (1 μg/ml) and salbutamol glucuronide (20 ng/ml) via a syringe pump and observation of the ESI response during injection of six different urine specimens into the HPLC-MS system.[28]

Liquid chromatography-tandem mass spectrometry

Different LC-MS/MS instruments were employed: For the direct quantification of salbutamol an Agilent 1100 LC Applied Biosystems API 2000 triple quadrupole MS (APCI) was used, screening for salbutamol glucuronide and recording product ion mass spectra from reference material was performed by an Agilent 1200 LC Applied Biosystems API 4000 Qtrap (ESI) and the determination of salbutamol glucuronide was conducted via an Agilent 1100 LC Applied Biosystems API 5500 Qtrap (ESI).

The LC was equipped with a Macherey-Nagel Pyramid Nucleodur C8 colum (4.6 x 70 mm, 5 μm particle size), and the eluents used were (1) 5 mM ammonium acetate buffer containing 0.1% of glacial acetic acid (pH = 3.5), and (2) acetonitrile. A gradient was employed from 0% B to 100% B within 8 min, and the column was re-equilibrated

at 0% B for 4.5 min. The flow rate was set to 800 µl/min. Positive ionization was accomplished at an interface temperature of 400 °C. The collision gas was nitrogen at a nominal pressure of 3.5 x 10^{-3} Pa (obtained from a K75-72 Whatman nitrogen generator) and collision offset voltages were optimized for each product ion.

Salbutamol was detected by means of characteristic product ions at *m/z* 166, *m/z* 148 and *m/z* 222, formed from the protonated molecule $[M+H]^+$ at *m/z* 240 by ESI utilizing the multiple reaction monitoring mode (MRM). The identification of salbutamol glucuronide was performed utilizing the ion transitions *m/z* 416–148, 416–398 and 416–222.

For quantification purposes the following ion transitions were used: salbutamol (*m/z* 240–148), **d_3**-salbutamol (*m/z* 243–169), salbutamol glucuronide (*m/z* 416–148) and **d_3**-salbutamol glucuronide (*m/z* 419–151).

Administration study urine samples

Ten asthmatic subjects, eight male elite athletes suffering from asthma and ten healthy non-asthmatic men participated in an open-label, crossover design study, performed by Backer *et al.* at the Bispebjerg Hospital, Copenhagen, Denmark.[29]

In the first part of the study, the subjects inhaled four times 0.2 mg of salbutamol (Ventoline® Diskos 0.2 mg). In the second part, the volunteers received twice 4 mg of salbutamol orally (Ventoline® tablet 4 mg). Urine samples were collected before administration and 4, 8, and 12 h after application of the drug. The total volume of each sample was recorded, and a 30-ml aliquot was stored frozen until shipment to the laboratory. After reception, the samples were stored frozen again until analysis. In total, 220 urine samples were analyzed. Written informed consent was obtained from all subjects. The protocol was approved by the local ethics committee. More details of the study design are presented by Elers *et al.*[29]

Statistical data evaluation

The measured concentrations of salbutamol and its glucuronide were corrected for specific gravity before statistical analyses were performed. Furthermore, all values were log-transformed in order to achieve approximate linearity of the excretion trends.

The data set was analyzed by fitting a linear mixed effects model. Time after application (TIME), type of application (APPL), and health state (STATUS) served as independent variables. TIME was converted to ordinal scale where three levels were defined: 0 through 4 (Time 1), > 4 through 8 (Time 2), and > 8 through 12 h after application (Time 3). APPL featured the levels inhalation (I) and oral application (O). The different health states were defined as healthy men (H), asthmatic (A) subjects and asthmatic athletes (AA).

Initially, purely additive effects of the three fixed predictors were assumed. Subsequently interaction effects between STATUS and APPL and between APPL and TIME were added. The resulting models were compared by likelihood ratio statistics. The subjects were assumed to feature randomly distributed intercepts, i. e. individually different concentrations between 0 and 4 h after application. Having found an appropriate model for the fixed effects, the slopes were tested for individual variability. Again, likelihood statistics was employed to compare the models.

Authentic doping control urine samples

Sixty-five doping control urine samples were analyzed, received in 2010 from national and international federations, taken in- and out-of-competition, indicating screening results for salbutamol between 15 and 600 ng/ml. Additionally, three specimens containing more than 1000 ng/ml salbutamol and reported as adverse analytical findings were analyzed. The specimens were stored frozen until analysis.

Results and discussion

Mass spectrometry

Product ion mass spectra generated from protonated molecules $[M+H]^+$ of salbutamol (*m/z* 240), **d_3**-salbutamol (*m/z* 243), salbutamol glucuronide (*m/z* 416) and **d_3**-salbutamol glucuronide (*m/z* 419), were recorded from reference material and generated at collision offset voltages of 30 eV (Figures 1a–1d).

In case of salbutamol, the neutral loss of a water molecule (−18 u) is observed, generating the fragment at *m/z* 222, followed by the elimination of isobutene (−56 u) yielding the ion at *m/z* 166. The most abundant ion results from the subsequent elimination of H_2O leading to *m/z* 148. For the internal standard **d_3**-salbutamol a similar fragmentation pattern is observed yielding the product ions at *m/z* 225, 169, and 151, accordingly.

The product ion mass spectrum of salbutamol glucuronide is composed of ions generated from losses of water molecules (*m/z* 398, 380, 306, 222, 204, 148, 130), elimination of isobutene (*m/z* 324, 166) and release of the glucuronide (−176 u) moiety (*m/z* 240, 222). The similar fragmentation pattern of the internal standard **d_3**-salbutamol glucuronide shows an addition of 3 u each to the particular ions. The location of the glucuronide moiety in the synthesized reference material is unknown, as depicted in Figures 1c and 1d.

The identification of salbutamol glucuronide in administration study urine samples and authentic doping control urine samples was performed according to the currently effective WADA Technical Document TD2010IDC.[30] The most suitable ion transitions *m/z* 416–148, 416–222 and 416–398 were utilized. The retention time of the target compound under the chosen conditions was 3.74 min (Figure 2). The existence of salbutamol glucuronide isomers cannot be excluded. The analytical determination is covered by the presented method, resulting from the coelution of the respective analytes.

Assay validation for salbutamol and salbutamol glucuronide

In order to test for assay suitability, the parameters specificity, LLOD, LLOQ, linearity, intra- and inter-day precision, recovery, and extraction efficiency were determined according to International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines[31] (Tables 1a and 1b).

Regarding specificity, the analysis of six different blank urine samples did not yield interfering signals at the expected retention time for salbutamol or salbutamol glucuronide. At the required signal-to-noise ratio of 3, the LLOD of salbutamol was estimated at 5 ng/ml and the corresponding LLOQ at 15 ng/ml. For salbutamol glucuronide the LLOD was determined with 2 ng/ml. The LLOQ was estimated at 5 ng/ml with good precision and recovery as presented in Table 1b.

Linear calibration curves were obtained for salbutamol (range: 250–2500 ng/ml; calibration equation of $y = 0.00189x + 0.161$ with $r = 0.9946$) and salbutamol glucuronide (range: 5–60 ng/ml; calibration equation of $y = 0.0423x + 0.0407$ with $r = 0.9978$). Linearity was proved by the Mandel test.[32]

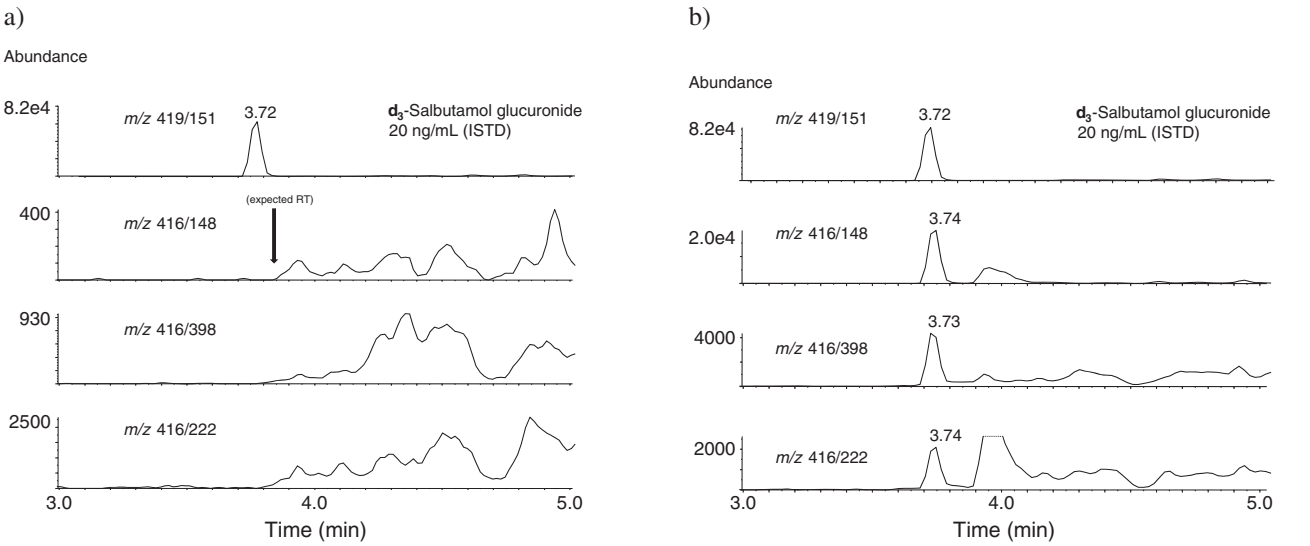

Figure 2. Extracted ion chromatograms of (a) blank urine containing the internal standard ($\mathbf{d_3}$-salbutamol glucuronide) only, and (b) urine specimen (administration study urine sample collected after oral ingestion of salbutamol) yielding approx. 6 ng/ml salbutamol glucuronide measured on an Applied Biosystems API 5500 Qtrap. The analyte salbutamol glucuronide was determined using the diagnostic ion transitions *m/z* 416–148, 416–398 and 416–222 employing multiple reaction monitoring (detection at retention time 3.74 min).

The intra-day and inter-day precisions were determined at three concentration levels and show variations of less than 5% for low (500 ng/ml), medium (1000 ng/ml), and high (2000 ng/ml) salbutamol concentration, respectively less than 7% for low (5 ng/ml), medium (20 ng/ml), and high (40 ng/ml) salbutamol glucuronide concentration. At three concentration levels (5, 20, 40 ng/ml) the recovery was determined by means of the measured calibration curve and ranged between 100 and 109%. The extraction efficiency was specified with 78%.

For salbutamol no suppression of the ESI response was observed, while for salbutamol glucuronide suppression effects of approx. 30% were detected. For compensation the use of an adequate internal standard (e.g. d3-salbutamol glucuronide) is recommended.

Administration study urine samples

Unconjugated salbutamol was detected in all urine samples collected following the inhalative and oral administration of therapeutic doses of salbutamol preparations.

Inhalation (4 x 0.2 mg salbutamol Ventoline® Diskos)

Urinary concentrations of salbutamol after inhalation ranged from 8 to 1029 ng/ml. Maximum values were obtained from 20 of 28 volunteers in samples collected 0–4 h after administration. Only one specimen (of a healthy non-asthmatic volunteer) showed a salbutamol concentration slightly above 1000 ng/ml, indicating a borderline situation. As provided on the Prohibited List,[1] in such case, the respective athlete would have to prove

Table 1a. Summary of assay validation for the direct quantification of salbutamol in human urine by means of LC-MS/MS

	Intra-day precision (n = 6 + 6 + 6)	Inter-day precision (n = 18 + 18 + 18)		
Concentration (ng/ml)	CV (%)	CV (%)	Recovery (%)	Mean values of the replicate (ng/ml)
500	2.4	3.4	97	486
1000	2.2	4.6	99	988
2000	3.2	3.7	100	2006

Table 1b. Summary of assay validation for the salbutamol glucuronide determination in human urine by means of LC-MS/MS

	Intraday precision (n = 6 + 6 + 6)	Interday precision (n = 18 + 18 + 18)	Recovery (n = 6 + 6 + 6)	Mean values of the replicate (n = 6 + 6 + 6)
Concentration (ng/ml)	CV (%)	CV (%)	(%)	(ng/ml)
5	4.8	5.9	100	5.0
20	4.4	6.1	105	21.0
40	5.7	4.6	109	43.6

(through a controlled pharmacokinetic study) that the abnormal result is the consequence of the use of a therapeutic dose of inhaled salbutamol.

No salbutamol glucuronide was detected in concentrations higher than 2 ng/ml (LLOD) in screening analysis of urine samples collected following inhalation.

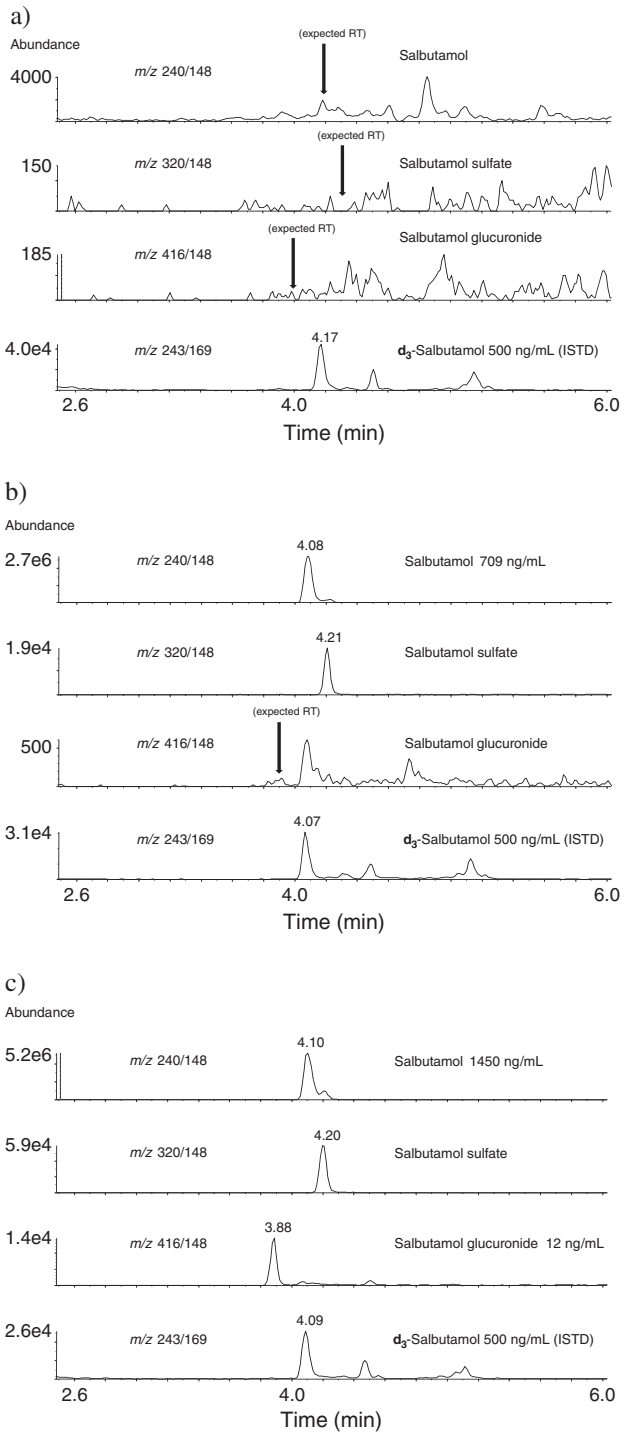

Figure 3. Extracted ion chromatograms of (a) blank urine containing the internal standard ($\mathbf{d_3}$-salbutamol) only, (b) urine specimen collected after inhalation of 0.8 mg salbutamol, and (c) urine specimen collected after oral ingestion of 8 mg salbutamol measured on an Applied Biosystems API 2000 triple quad MS.

Oral ingestion (8 mg salbutamol Ventoline® tablet)

Salbutamol concentrations after oral administration were much higher than those after inhalation. Most of the volunteers (22 of 28) reached peak levels between 0 and 8 h post administration with a maximum value of 9320 ng/ml for an asthmatic subject. For most volunteers the threshold level of 1000 ng/ml was exceeded at least in one urine sample within the study. In 26 out of 82 specimens salbutamol glucuronide was detected with a peak value of 63 ng/ml, most of them were obtained between 4 and 12 h after application.

The percentage of urinary salbutamol glucuronide compared to unconjugated salbutamol after oral ingestion ranged less than 3%.

Extracted ion chromatograms of urine specimen collected after inhalative and oral ingestion of salbutamol are presented in Figure 3.

Statistical evaluation in administration study urine samples

Starting from the additive model, introduction of an interaction term between STATUS and APPL resulted in a highly significant improvement of the fit ($p < 0.001$). By contrast, assuming an additional interaction between TIME and APPL did not result in further improvement of the model. The latter term has been omitted from the final model.

Table 2 summarizes the fixed parameters of the model found to be appropriate.

Random effects could be restricted to individual variation between intercepts. On the log-scale, this corresponds to largely parallel excretion curves (Figure 4).

Authentic doping control urine samples

Two of 65 doping control urine samples indicating screening results for salbutamol at 250 and 500 ng/ml showed salbutamol glucuronide concentrations between LLOD and LLOQ (estimated at 4 and 5 ng/ml).

Three adverse analytical findings for salbutamol with confirmed concentrations of 1437, 1478 and 2229 ng/ml gave rise to corresponding salbutamol glucuronide values of 8, 10, and 15 ng/ml. The percentage of salbutamol glucuronide compared to salbutamol was calculated to be less than 1%.

Conclusion

The presented assay is a suitable tool for the rapid determination of salbutamol and its glucuronic acid conjugate in human urine and represents an alternative to existing procedures since time consuming hydrolysis or derivatization steps were omitted.

Table 2. Fixed parameters of the fitted linear mixed effects model

	Value	Std. Error	DF	t-value	p-value
(Intercept)	5.56	0.1486	131	37.4	0.0000
Status AA	0.41	0.2085	25	2.0	0.0591
Status H	0.89	0.1955	25	4.5	0.0001
Appl 0	2.42	0.1282	131	18.9	0.0000
Time 2	−0.34	0.0939	131	−3.6	0.0005
Time 3	−0.99	0.0940	131	−10.6	0.0000
Status AA x Appl 0	−0.26	0.1950	131	−1.3	0.1871
Status H x Appl 0	−0.76	0.1904	131	−4.2	0.0000

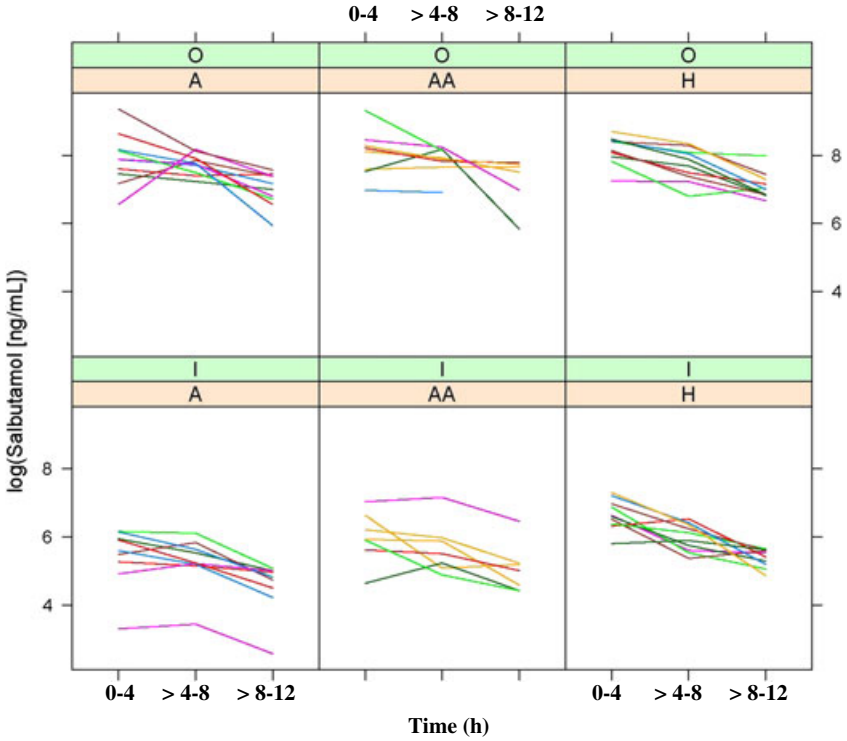

Figure 4. Effects of administration form and volunteer group on specific gravity adjusted urinary salbutamol concentration in administration urine samples. The salbutamol values are presented in logarithmic form.
Administration form O: oral ingestion I: inhalation
Volunteer group A: asthmatic subjects AA: asthmatic athletes H: healthy men

The simultaneous consideration of the unconjugated salbutamol and its glucuronide is of importance because the WADA regulations require the consideration of both for the threshold value. The present investigation showed in some cases a percentage of salbutamol glucuronide compared to unconjugated salbutamol up to approx. 3%. Based on these results a reconsideration of the WADA rules concerning the quantification of salbutamol would be desirable.

The excretion of salbutamol glucuronide in human urine following administration of salbutamol has been proven and is reported for the first time.

Acknowledgements

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References

[1] World Anti-Doping Agency. The 2011 Prohibited List. International Standard. Available at: http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/To_be_effective/WADA_Prohibited_List_2011_EN.pdf [16 December 2010].
[2] World Anti-Doping Agency. Decision Limits for the Confirmatory Quantification of Threshold Substances. Available at: http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/WADA_TD2010DLv1.0_Decision%20Limits%20for%20the%20Confirmatory%20Quantification%20of%20Threshold%20Substances_May%2008%202010_EN.doc.pdf [20 July 2010].
[3] R. Berges, J. Segura, R. Ventura, X. De la Torre, M. Mas, M. Farre, K. D. Fitch, A. R. Morton, M. Ueki, in Recent Advances in Doping Analysis (6), (Eds: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke), Sport & Buch Strauß, Köln, **1998**, pp. 405–419.
[4] R. Ventura, J. Segura, R. Berges, K. D. Fitch, A. R. Morton, S. Berruezo, C. Jimenez, *Ther. Drug Monit.* **2000**, *22*, 277.
[5] R. Berges, J. Segura, X. De la Torre, R. Ventura, *J. Chromatogr. B* **1999**, *723*, 173.
[6] R. Berges, J. Segura, R. Ventura, K. D. Fitch, A. R. Morton, M. Farre, M. Mas, X. De la Torre, *Clin. Chem.* **2000**, *46*, 1365.
[7] G. Forsdahl, G. Gmeiner, *J. Separ. Sci.* **2004**, *27*, 110.
[8] A. de la Cal, G. Munoz, S. Vargas, J. A. Munoz-Guerra, F. A. Rodriguez, C. Rodriguez, in Recent Advances in Doping Analysis (13), (Eds: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck), Sportverlag Strauß, Köln, **2005**, pp. 487–490.
[9] J. Zhang, Y. Xu, X. Di, M. Wu, *J. Chromatogr. B* **2006**, *831*, 328.
[10] M. K. Henze, H. Spahn-Langguth, W. Schänzer, in Recent Advances in Doping Analysis (9), (Eds: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke), Sport & Buch Strauß, Köln, **2001**, pp. 29–33.
[11] M. K. Henze, Screening auf β2-Sympathomimetika in der Dopinganalytik Martin-Luther-Universität, Halle, **2001**.
[12] I. Bellahn, E. Nolteernsting, G. Opfermann, W. Schänzer, in Recent Advances in Doping Analysis (6), (Eds: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke), Sport & Buch Strauß, Köln, **1998**, pp. 489–496.
[13] C. Schweizer, P. Mangin, M. Saugy, in Recent Advances in Doping Analysis (11), (Eds: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck), Sport & Buch Strauß, Köln, **2003**, pp. 289–293.
[14] N. Halatcheva, E. Daneva, in *Recent Advances in Doping Analysis (8)*, (Eds: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke), Sport & Buch Strauß, Köln, **2000**, pp. 189–196.
[15] M. Donike, H. Geyer, A. Gotzmann, M. Kraft, F. Mandel, E. Nolteernsting, G. Opfermann, G. Sigmund, W. Schänzer, J. Zimmermann, in International Athletic Foundation World Symposium on Doping in Sport, (Eds: P. Bellotti, G. Benzi, A. Ljungqvist), International Athletic Foundation, Florence, **1987**, pp. 53–80.
[16] H. Geyer, W. Schänzer, U. Mareck-Engelke, E. Nolteernsting, G. Opfermann, in Recent Advances in Doping Analysis (5), (Eds: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke), Sport & Buch Strauß, Köln, **1997**, pp. 99–101.

[17] U. Mareck, M. Thevis, S. Guddat, A. Gotzmann, M. Bredehöft, H. Geyer, W. Schänzer, in Recent Advances in Doping Analysis (12), (Eds: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck), Sport & Buch Strauß, Köln, **2004**, pp. 65–68.
[18] A. Gotzmann, M. Donike, in Proceedings, (Eds: M. Donike, H. Geyer, A. Gotzmann, U. Mareck-Engelke, S. Rauth), Sport & Buch Strauß, Köln, **1992**, pp. 281–284.
[19] G. Gmeiner, T. Geisendorfer, J. Kainzbauer, M. Nikolajevic, in Recent Advances in Doping Analysis (9), (Eds: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck-Engelke), Sport & Buch Strauß, Köln, **2001**, pp. 43–51.
[20] M. Thevis, G. Sigmund, W. Schänzer, in Recent Advances in Doping Analysis (11), (Eds: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck), Sport & Buch Strauß, Köln, **2003**, pp. 303–307.
[21] M. Thevis, W. Schänzer, *J. Chromatogr. Sci.* **2005**, *43*, 1.
[22] U. Mareck, N. Haenelt, M. K. Parr, S. Guddat, A. Thomas, M. Thevis, W. Schänzer, in Recent Advances in Doping Analysis (15), (Eds: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck), Sport & Buch Strauß, Köln, **2007**, pp. 87–96.
[23] T. Kuuranne, M. Kurkela, M. Thevis, W. Schänzer, M. Finel, R. Kostiainen, *Drug Metab. Dispos.* **2003**, *31*, 1117.
[24] T. Kuuranne, A. Leinonen, W. Schänzer, M. Kamber, R. Kostiainen, M. Thevis, *Drug Metab. Dispos.* **2008**, *36*, 571.
[25] L. E. Martin, J. C. Hobson, J. A. Page, C. Harrison, *Eur. J. Pharmacol.* **1971**, *14*, 183.
[26] C. Lin, Y. Li, J. Mcglotten, J. B. Morton, S. Symchowicz, *Drug Metab. Dispos.* **1977**, *5*, 234.
[27] S. Kromidas, Validierung in der Analytik, Wiley-VCH, Weinheim, **1999**.
[28] T. M. Annesley, *Clin. Chem.* **2003**, *49*, 1041.
[29] J. Elers, L. Pedersen, J. Henninge, T. K. Lund, P. Hemmersbach, K. Dalhoff, V. Backer, *Med. Sci. Sport Exer.* **2010**, *42*, 244.
[30] World Anti-Doping Agency, Identification Criteria for Qualitative Assays Incorporating Column Chromatography and Mass Spectrometry. Available at: http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-IS-Laboratories/WADA_TD2010IDCRv1.0_Identification%20Criteria%20for%20Qualitative%20Assays_May%2008%202010_EN.doc.pdf [1 September 2010].
[31] Validation of Analytical Procedures: Text and Methodology Q2 (R1). Available at: http://www.ich.org/LOB/media/MEDIA417.pdf [27 November 2008].
[32] W. Gottwald, Statistik für Anwender, Wiley-VCH, Weinheim, **2000**.