U.Mareck, N.Haenelt, M.K.Parr, S.Guddat, A.Thomas, M.Thevis and W.Schänzer

Direct Quantification of Salbutamol in Human Urine by Means of LC-MS/MS

Institute of Biochemistry, German Sport University Cologne, Germany

Abstract

According to WADA rules salbutamol concentrations in urine greater than 1 µg/ml are defined as a doping violation. Concentrations greater than 500 ng/ml should be reported as an adverse finding relative to use of a ß2 agonist (WADA Technical Document – MRPL revised version 2007). The threshold concentration is based on the sum of the glucuronide conjugate and free drug concentrations.

Because of the time consuming effort of sample preparation, a relatively high concentration of salbutamol in the urine samples and adequate LC behaviour of salbutamol an alternative approach for quantitative determination of salbutamol in doping control samples was developed utilizing LC-MS/MS. The urine samples are fortified with deuterated salbutamol and without any further sample preparation, aliquots of 10 µl are injected into the LC-MS/MS instrument. Lower limit of detection was 20 ng/ml, interday precisions 14.1% for low (200 ng/mL), 7.1% for medium (500 ng/mL) and 4.8% for high (1000 ng/mL) concentration. The intermediate precision was 7.7% for the reporting threshold 500 ng/mL and 11.8% for the 1000 ng/mL threshold, respectively.

Introduction

According to WADA rules urinary salbutamol concentrations (free and glucuronide) greater than 1 µg/mL are defined as a doping violation (1,2). Concentrations greater than 500 ng/mL should be reported as an adverse analytical finding beginning on 1st of January 2007. The threshold is based on considerations that concentrations below 500 ng/mL reflect normal therapeutic use of inhaled salbutamol.

Numerous articles on the analysis of salbutamol in urine have been published. Extensive investigations about the discrimination of 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 by means of GC-MS and LC-MS following solid phase extraction (SPE) sample preparation was well investigated (7-9). Experiments concerning the stability of salbutamol in native urinary matrix were performed and it was demonstrated that the concentration decreases 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 strong variations in excretion study urine samples (12-14). Hence, the established threshold was based on the sum of glucuronide conjugate and free drug concentrations.

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).
Inhouse 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 and the behavior 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 labeled salbutamol was evaluated in accordance with earlier studies.
The quantitative determination of caffeine by direct injection of urine with HPLC-UV has been performed since 1993 in the Cologne Doping Control Laboratory (18). Gmeiner *et al* presented a LC-method for the quantification of ephedrines in urine without sample preparation by trapping the analytes on a precolumn and subsequent analysis via HPLC/DAD (19).
Thevis *et al* described the rapid quantitative analysis of ephedrines by liquid chromatography – tandem mass spectrometry without sample preparation (20,21). Based on this method an alternative approach for quantitative determination of salbutamol in doping control samples was developed. The urine samples are fortified with deuterated salbutamol and injected - without any further sample preparation - into the LC-MS/MS instrument. Detection limit, precision and specificity were established. Additionally the presence of salbutamol glucuronide and salbutamol sufate in administration urine samples was checked under the same conditions.

Experimental

Salbutamol

Salbutamol sufate (Salbulair 4®) was obtained from 3M Medica (Borken, Germany) and Salbulair® N Dosieraerosol was purchased from IVAX Pharma (Neuss, Germany). Salbutamol sufate extracted from human urine was characterized by nuclear magnetic resonance (NMR) spectroscopy and obtained from Bayer Industries (Leverkusen, Germany). Salbutamol glucuronide was obtained from an urine excretion study in rats.

Chemicals and reagents

All solvents and reagents were of analytical grade. Salbutamol was bought from Sigma-Aldrich (Taufkirchen, Germany). D6-salbutamol (RIVM, European Union Reference Laboratory, Bilthoven, The Netherlands) was used as internal standard.
All solutions and buffers were prepared with deionized water (Water Lab System, Millipore, Eschborn, Germany).

Sample preparation

The sample preparation was modified from an assay for quantitative caffeine determination by direct injection of urine using HPLC-UV described by Gotzmann and Donike (18). Urine samples (1 mL) were fortified with the internal standard d_6-salbutamol (500 ng/mL) and without any further sample preparation, aliquots of a volume of 10 µl were injected into the LC-MS/MS instrument.

Liquid chromatography/tandem mass spectrometry

All samples were analyzed by LC-MS/MS employing an Agilent 1100 series liquid chromatograph coupled to an Applied Biosystems API 2000 triple quadrupole mass spectrometer utilizing atmospheric pressure chemical ionization (APCI). The liquid chromatograph was equipped with a Machery & Nagel Nucleodur® C-18 Pyramid column (70 x 4 mm, 5µm particle size), and the eluents used were A: 5 mM ammonium acetate buffer containing 0.1% of glacial acetic acid (pH = 3.5), and B: acetonitrile. A gradient was used from 0% B to 100% B within 7 min, and the column was re-equilibrated at 0% B for 1 min. The flow rate was set to 800 µL/min.
Positive ionisation was accomplished by atmospheric pressure chemical ionisation (APCI) at an interface temperature of 400°C. Declustering potential was set to 15 V, and collision energies were optimized individually for each ion transition. Nitrogen (obtained from a K75-72-727 Whatman nitrogen generator) was used as collision gas at a collision cell pressure of 2.93e-3 Pa.

Ion transitions *m/z* 240/148 (salbutamol) and *m/z* 246/148 (d_6-salbutamol) were used for quantifications.

Administration urines

An excretion study was performed in agreement with the ethical commission of the German Sport University Cologne by a healthy female volunteer who has given written consent. Four mg of salbutamol sufate (Salbulair 4®) was orally administered. Aliquots of each urine sample were collected for 48 hours and, thereafter, morning urine samples over a time period of 3 days. In total, urine samples were collected for 5 days.

A urine sample of a patient utilizing Salbulair® N dosieraerosol for the treatment of exercise enduced asthma was obtained approximately 2 h after administration of one regular dosis (2 puffs containing 0.24 mg salbutamol sulfate).

Assay validation

Calibration curve

A calibration curve for salbutamol was generated using blank urine spiked at 200, 400, 600, 800, 1000, 1200, 1400, 1600, 1800 and 2000 ng/mL.

The peak area ratios of analyte and ISTD were utilized to calculate the correlation coefficient, intercept and slope.

Lower limit of detection

The lower limit of detection (LLOD) was defined as the “lowest content that can be measured with reasonable statistical certainty” (22) at a signal-to noise (S/N) ratio $\geq$3. Ten blank urine samples spiked with the internal standard (ISTD) only, and ten blank urine specimens fortified with 20 ng of salbutamol per mL, were prepared and analyzed according to the established protocol.

Interday precision

On three consecutive days, ten urine samples of low (200 ng/mL), medium (500 ng/mL) and high (1000 ng/mL) concentrations of salbutamol were prepared, analyzed randomly, and the assay precision was calculated for each concentration level.

Specificity

Ten different blank urine specimens were prepared as described above in order to probe for interfering peaks in the selected ion chromatograms at the expected retention time of salbutamol.

Intermediate precision

The urine samples, which were also used for the determination of specificity, were fortified with 500 and 1000 ng/mL of salbutamol, analyzed and the intermediate precision was calculated for both threshold values.

Results and Discussion

Mass spectrometry

Product ion mass spectra generated from protonated molecules $[M+H]^+$ of salbutamol (*m/z* 240) and d_6-salbutamol (*m/z* 246) were recorded from reference material and generated at collision offset voltages of 25 eV, as depicted in Figs. 1-2.

Protonation of the analytes is likely at the nitrogen of the ethanolamine side-chain.

In salbutamol the neutral loss of a water molecule (-18 u) is observed, generating the fragment at *m/z* 221, followed by the elimination of isobutene (-56 u) yielding the ion at *m/z* 166. The most abundant ion results from the further elimination of H_2O leading to *m/z* 148.

For the internal standard d_6-salbutamol a similar fragmentation pattern is observed. The elimination of water (*m/z* 228) is followed by the elimination of isobutene bearing either six deuteria and two hydrogens (-62 u) or five deuteria and three hydrogens (-61 u) generating the product ions at *m/z* 166 and 167, respectively. The subsequently release of water is leading to the ions at *m/z* 148 and 149.

Assay validation

Calibration curve

A linear calibration curve was obtained in urine over a range of 200 - 2000 ng/mL. The calibration equation was $y = 0.0079\ x + 0.0907$ with $r = 0.9874$.

Linearity was proven by the F-test and homoskedasticity by the Breusch-Pagan-Test.

Lower limit of detection

The average background noise plus a three-fold standard deviation was calculated for ten blank samples at the respective retention time in specific extracted ion transition chromatograms, and a S/N ratio greater than 3 used as the decision criterion. The LLOD was determined at 20 ng/mL.

Interday precision

The interday precision was determined at three concentrations and was 14.1% , 7.1%, and 4.8% for low (200 ng/ml), medium (500 ng/ml), and high (1000 ng/ml) concentrations, respectively.

Specificity

In all ten blank urine specimens, interfering signals were not observed at the retention time of salbutamol.

Intermediate precision

The intermediate precisions were less than 15% for the concentration levels 500 ng/mL (7.7%) and 1000 ng/mL (11.8%).

Administration studies

The presented method was checked by analyzing urines after inhalations and oral administrations.

As shown in Figure 3 and 4 no salbutamol glucuronide was detectable. For comparison purposes a reference was obtained from a rat excretion study. Both urine specimen contain salbutamol and its sulfate conjugate, whereas – as expected - the oral administration urine sample shows higher concentrations of the analytes than the urine sample after inhalative application. According to WADA regulations the salbutamol threshold is based on the sum of the glucuronide conjugate and free drug. Resulting from the absence of salbutamol glucuronide in human urine the presented method is recommended for confirmation purposes.

Conclusion

For the direct quantification of salbutamol in human urine suitable ion transitions are *m/z* 240/148 (salbutamol) and *m/z* 246/148 (ISTD d_6-salbutamol). The additional detection of salbutamol sulfate (*m/z* 320/222) is possible (Figure 3 - 4).

Acknowledgements

The authors wish to thank the Manfred Donike Institute (MDI) for support.

References

1. World Anti-Doping Agency. The 2007 Prohibited List. International Standard, Montreal (2007) http://www.wada-ama.org/rtecontent/document/2007_List_En.pdf (access date 06.02.2007)
2. World Anti-Doping Agency: WADA Technical Document – TD2004 MRPL. Minimum Required Performance Limits for Detection of Prohibited Substances. http://www.wada-ama.org/rtecontent/document/perf_Limits_2.pdf (access date 06.02.2007)
3. Bergés R, Segura J, Ventura R, de la Torre X, Mas M, Farré M, Fitch KD, Morton AR, Ueki M. (1998) Oral versus Inhaled Salbutamol: Is Distinction Possible by Urine Analysis? In: Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U (eds) *Recent Advances in Doping Analysis (6),* Sport und Buch Strauß, Köln. pp 405-419.
4. Ventura R, Segura J, Bergés R, Fitch KD, Morton AR, Berruezo S, Jimenez C. (2000) Distiction of inhaled and oral salbutamol by urine analysis using conventional screening procedures for doping control. *Ther Drug Monit.* 22(3), 277-82.
5. Bergés R, Segura J, de la Torre X, Ventura R. (1999) Analytical methodology for enantiomers of salbutamol in human urine for application in doping control. *J Chromatogr B Biomed Sci Appl.* 19; 723 (1-2):173-84.
6. Bergés R, Segura J, Ventura R, Fitch KD, Morton AR, Farré M, Mas M, de la Torre X. (2000) Discrimination of prohibited oral use of salbutamol from authorized inhaled asthma treatment. *Clinical Chemistry* 46:9; 1365-1375.
7. Forsdahl G, Gmeiner G. (2004) Quantification and stability of salbutamol in human urine. *J Sep Sci.* 27(1-2):110-4.
8. De la Cal A, Munoz G, Vargas S, Munoz-Guerra JA, Rodriguez FA, Rodriguez C. (2005) A fast quantification of Salbutamol using automated SPE and liquid chromatography mass spectrometry. In: Schänzer W, Geyer H, Gotzmann A, Mareck U (eds) *Recent Advances in Doping Analysis (13),* Sport und Buch Strauß, Köln. pp 487-490.
9. Zhang J, Xu Y, Di X, Wu M. (2006) Quantitation of salbutamol in human urine by liquid chromatography-electrospray ionization mass spectrometry. *J Chromatogr B* 831(1-2):328-32.
10. Henze MK, Spahn-Langguth H, Schänzer W. (2001) Reactions of Salbutamol and Reproterol with urine matrix components – preliminary results. In: Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U (eds) *Recent Advances in Doping Analysis (9),* Köln. pp 29-33.
11. Henze MK. (2001) Screening auf ß2-Sympathomimetika in der Dopinganalytik. Entwicklung und Erprobung eines gaschromatographisch massenspektrometrischen Analyseverfahrens für Human-Urin. *Dissertation*, Shaker Verlag, Aachen, Germany.
12. Bellahn, I, Nolteernsting E, Opfermann G, Schänzer W. (1998) Preliminary results of excretion studies with Salbutamol. In: Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U (eds) *Recent Advances in Doping Analysis (6),* Köln. pp 489-496.
13. Schweizer C, Mangin P, Saugy M. (2003) High amount of Salbutamol: A case report. In: Schänzer W, Geyer H, Gotzmann A, Mareck U (eds) *Recent Advances in Doping Analysis (11),* Sport und Buch Strauß, Köln. pp 289-293.
14. Halatcheva N, Daneva E. (2000) Some aspects of investigation of extraction, derivatisation and excretion of Terbutaline and Salbutamol. In: Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U (eds) *Recent Advances in Doping Analysis (8),* Sport und Buch Strauß, Köln. pp 189-196.

15. Donike M, Geyer H, Gotzmann A, Kraft M, Mandel F, Nolteernsting E, Opfermann G, Sigmund G, Schänzer W, Zimmermann J. (1988) Dope analysis. In: Bellotti P, Benzi G, Ljungquist A (eds). *Official Proceedings of the International Athletic Foundation World Symposium on Doping in Sport*, Florence. pp 53-87.
16. Geyer H, Schänzer W, Mareck-Engelke U, Nolternsting E, Opfermann G. (1998) Screening procedure for anabolic steroids – the control of the hydrolysis with deuterated androsterone glucuronide and studies with direct hydrolysis. In: Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U (eds). *Recent Advances in Doping Analysis (5)*, Sport und Buch Strauß, Köln. pp 99-101.
17. Mareck U, Thevis M, Guddat S, Gotzmann A, Bredehöft M, Geyer H, Schänzer W. (2004) Comprehensive sample preparation for anabolic steroids, glucocorticosteroids, beta-receptor blocking agents, selected anabolic androgenic steroids and buprenorphine in human urine. In: Schänzer W, Geyer H, Gotzmann A, Mareck U (eds) *Recent Advances in Doping Analysis (12)*, Sport und Buch Strauß, Köln. pp 65-68.
18. Gotzmann A, Donike M. (1993) Quantitative caffeine determination by direct injection of urine. In: Donike M, Geyer H, Gotzmann A, Mareck-Engelke U, Rauth S (eds) *Proceedings*, Sport und Buch Strauß, Köln. pp 281-284.
19. Gmeiner G, Geisendorfer T, Kainzbauer J, Nikolajevic M. (2001) Quantification of ephedrines in urine without sample preparation. In: Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U (eds) *Recent Advances in Doping Analysis (9)*, Sport und Buch Strauß, Köln. pp 43-51.
20. Thevis M, Sigmund G, Schänzer W. (2003) Confirmation of ephedrines – Comparison between GC-MS and LC-MS/MS. In: Schänzer W, Geyer H, Gotzmann A, Mareck U (eds) *Recent Advances in Doping Analysis (11)*, Sport und Buch Strauß, Köln. pp 303-307.
21. Thevis M, Schänzer W. (2005) Examples of doping control analysis by liquid chromatography – tandem mass spectrometry: ephedrines, ß-receptor blocking agents, diuretics, sympathomimetics and cross-linked hemoglobins. *J Chromatogr Sci.* 43(1):22-31.
22. Kromidas S. *Validierung in der Analytik.* Wiley-VCH: Weinheim, 1999.
23. Thevis M, Opfermann G, Schänzer W. (2003) Liquid chromatography/electrospray ionization tandem mass spectrometric screening and confirmation methods for ß2-agonists in human or equine urine. *J. Mass Spectrom.* **38**: 1197-1206.

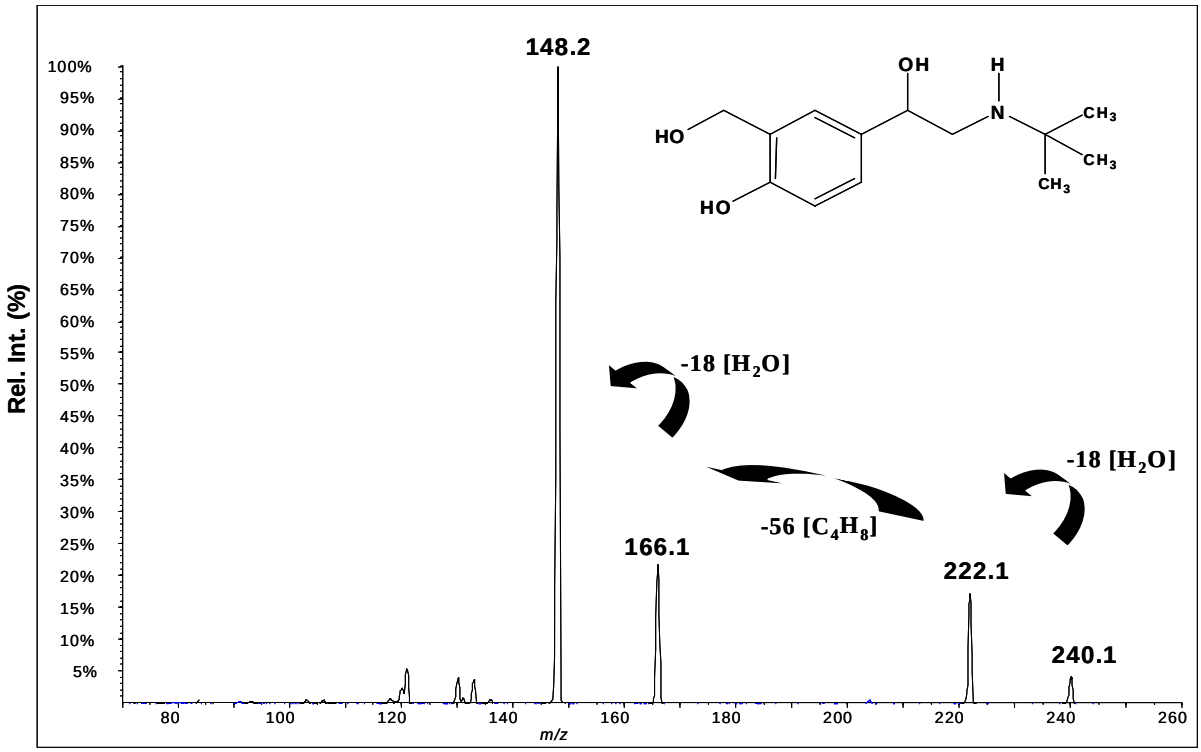


Figure 1: Product ion mass spectrum of salbutamol ($[M+H]^+$ = 240), recorded on an Applied Biosystems API 3200 triple quadrupole mass spectrometer using a CE of 25 eV.

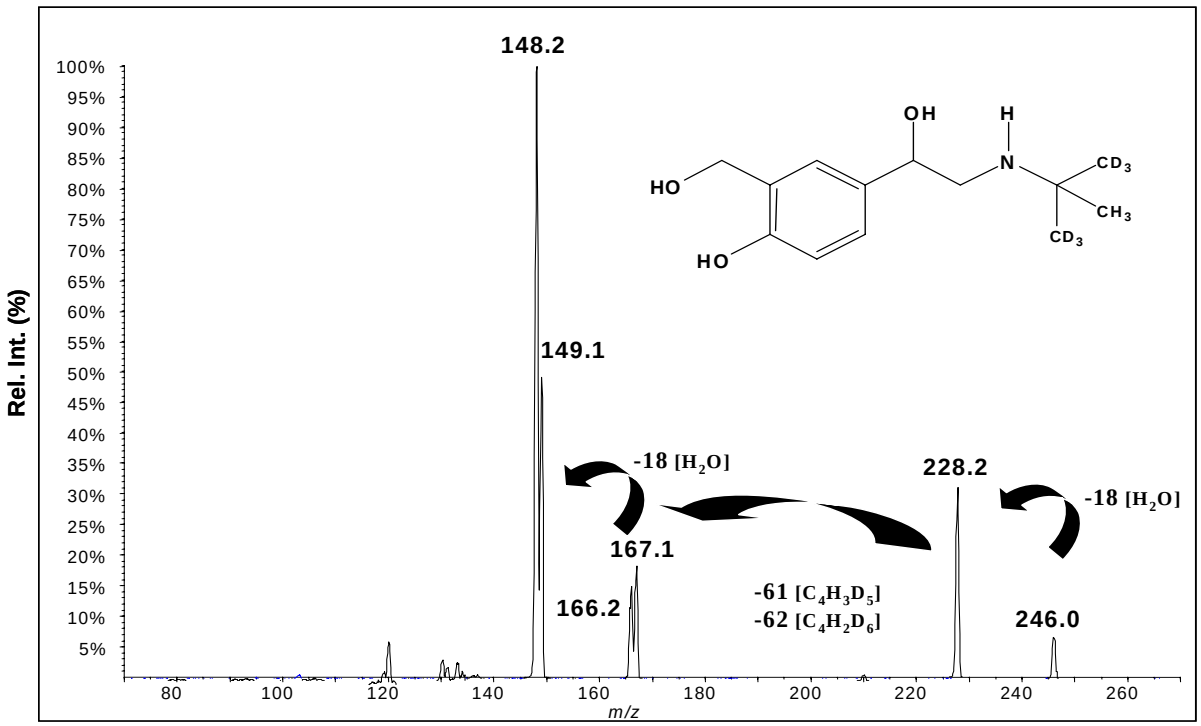
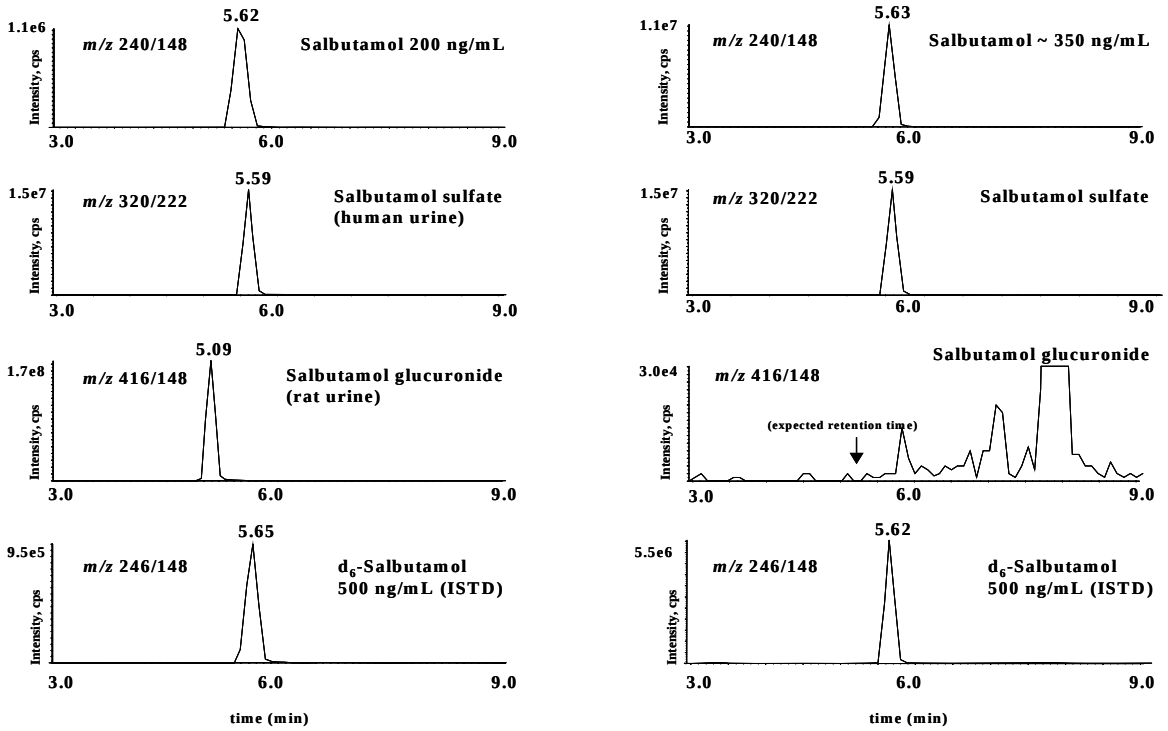


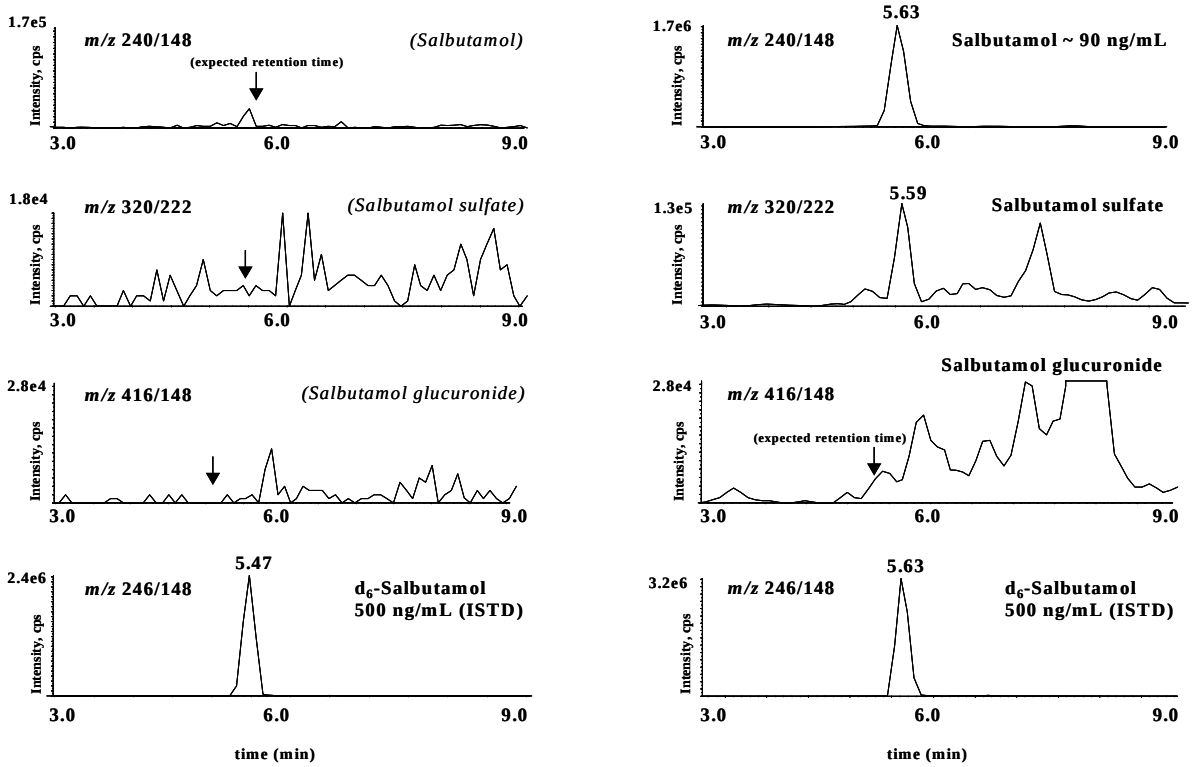
Figure 2: Product ion mass spectrum of d_6-salbutamol ($[M+H]^+$ = 246), recorded on an Applied Biosystems API 3200 triple quadrupole mass spectrometer using a CE of 25 eV.

Figure 3



Left: (standard) urine spiked with 200 ng/mL of salbutamol, salbutamol sulfate (excretion study reference standard, extracted from human urine), salbutamol glucuronide (excretion study rat urine), internal standard d_6-salbutamol (500 ng/mL).
Right: salbutamol excretion study urine specimen (oral application of salbutamol sulfate)

Figure 4



Left: blank urine with internal standard d_6-salbutamol (500 ng/mL)
Right: salbutamol excretion study urine sample (inhalation of salbutamol sulfate)