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High Resolution Quantitative Analysis of Beta-2-Agonists in Human Urine by LC/MS Using the Agilent LC/MSD TOF

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Introduction

Beta-2-agonists such as clenbuterol, mabuterol, bambuterol, and other sympathomimetic agents are commonly used for the treatment of pulmonary diseases such as asthma bronchial. In 1992, sympathomimetics were added to the list of prohibited substances by the World Anti-Doping Agency (WADA). They are classified as stimulants and anabolic agents with specific regulations in terms of competition and out-of-competition testing.

An LC/MS method has been developed for the sensitive qualitative and quantitative analysis of clenbuterol, mabuterol, bambuterol, and ractopamine to demonstrate the utility of an orthogonal axis electrospray time-of-flight mass spectrometer (oa-ESI-TOF) for doping control analysis.

Experimental

All LC/MS experiments were performed using an Agilent LC/MSD TOF mass spectrometer coupled to an Agilent 1100 Series LC system.
The TOF was operated with an orthogonal electrospray source in positive ion mode. A gradient method was used for chromatography. Clenpenterol was used as an internal standard for all analytes. Method conditions are given in Table 1.

Sample preparation

Samples were prepared according to the protocol established by the Institute of Biochemistry, German Sport University. Using this protocol, to a volume of 5 ml of urine, 300 µl of 10 M hydrochloric acid and 250 ng of clenpenterol were added. The sample was incubated at 80° C for 45 min. After cooling to ambient temperature, 5 ml of *tert.*-butyl methyl ether was added, the mixture was shaken for 15 min, centrifuged at 620*g* for 5 min, and the organic layer was discarded. To the remaining aqueous phase, 0.65 ml of 5M aqueous KOH, 1 ml of *tert.*-butanol, 500 mg of a mixture of K_2CO_3 and $NaHCO_3$ (2:1,w/w), 2 g of NaCl, and 5 ml of *tert.*-butyl methyl ether were added. The mixture was shaken for 15 min and centrifuged at 620*g* for 10 min. The organic layer was transferred to a fresh tube and evaporated to dryness at 50° C under vacuum. The dry residue was dissolved in 1 ml of 0.06M HCl, transferred to HPLC vials, and 20 µl were injected into the LC/MS system.

Chemicals:

hydrochloric acid 32% (e.g. Merck, 100319)
tert.-butanol
potassium hydroxide 85%
potassium carbonate (p.a.)
sodium hydrogencarbonate (anhydrous, p.a.)
sodium chloride (Merck)
tert.-butyl methyl ether (distilled before use), KMF, St. Augustin, Germany
sodium acetate (anhydrous, p.a.), Sigma

Results and Discussion

Several beta-2-agonists need to be identified in urine samples for doping control purposes in sports. The structures of the compounds used to demonstrate this methodology are given in Figure 1. A liquid-liquid extraction was used to extract the beta-2-agonists from the urine matrix. The time-of-flight mass spectrometer was used for identification with accurate mass

measurement and to quantify the analytes under control. This instrument only operates in full scan mode and this allows detection of any ionizable compound in the mass range. No special method setup was necessary for the mass spectrometer. This allows to add more analytes without changing the acquisition method.

Quantitation was performed by selecting the quasi-molecular ion to create extracted ion chromatograms (EICs) for each analyte and internal standard. Figure 2 displays example EICs from a 2 ng/ml spiked urine sample. Figure 3 shows calibration curves based on spiked urine samples as well as the statistics in Table 2. The WADA list of prohibited substances in sports gives includes detection limits for some of the beta-2-agonists. Clenbuterol and salbutamol are referred to as anabolic agents. For the latter a threshold of 1 µg/ml has been established. The capability shown here is well below those levels.

Identification was performed by using an automated empirical formula search routine. Added to the runtime worklist, this allows for an automated empirical formula calculation post acquisition reported in html and a comma separated value spreadsheet for further manipulation. Figure 4 displays a plot showing all calculated mass accuracy for standards as well as spiked urine samples. It shows that the system is able to measure routinely mass accuracy of better than 3 ppm in both standards and sample matrix.

Conclusions

An LC/MS method was developed for the qualitative and quantitative measurement of beta-2-agonists in human urine using the Agilent LC/MSD TOF system. The limit of quantitation for bambuterol, clenbuterol, mabuterol and ractopamin was at 2 ng/ml in human urine well below control levels. In addition, the ability to measure exact mass to accuracies better than 3 ppm at all levels and in sample matrix has been demonstrated.

Table 1: Conditions for LC/MS Analysis

HPLC	Agilent 1100
Flow Rate:	0.3 ml/min
Gradient:	Zorbax XDB-C18, 50mm x 2.1 mm, 3,5 µm
	A: Water + 5mM NH4OAC + 0.1% acetic acid
	B: Acetonitrile + 0.1% acetic acid
	0 min – 7 min: 5% A – 80% A; 7 min – 9 min: 80%
Injection:	20 µl out of 1000 µl

Agilent LC/MSD TOF system

Ionization mode:	Positive ESI
Nebulizer Pressure:	45 psi
Drying gas flow:	11 l/min
Drying gas temperature:	350 °C
Full Scan Mode	100 amu – 1000 amu
Automatic Reference	Ion 122 and Ion 922

QUANTITATION

Bambuterol	EIC of 368.20750 – 368.22959,Ret-Time: 6.3 min
Clenbuterol	EIC of 277.07858 - 277.09520, Ret-Time: 5.8 min
Mabuterol	EIC of 311.10392 - 311.12258, Ret-Time: 6.45 min
Ractopamin	EIC of 302.16600 - 302.18413, Ret-Time: 5.35 min
Clenpenterol(ISTD)	EIC of 291.09381 - 291.11128 +
	EIC of 293.09080 - 293.10839, Ret-Time: 6.4 min

Calibration Curves:	All weighted 1/x

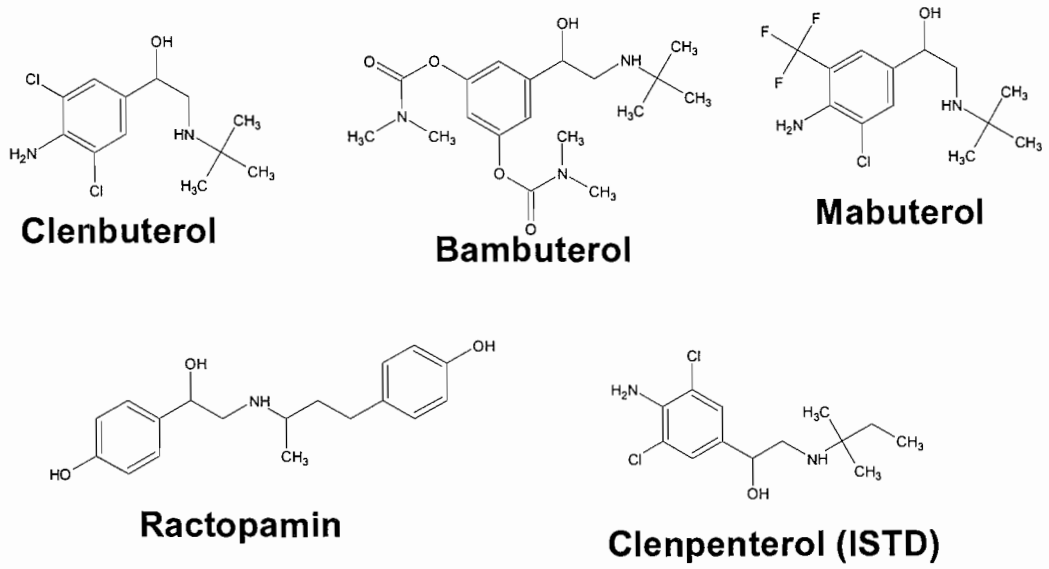

Figure 1. Chemical structures of the investigated beta-2-agonists

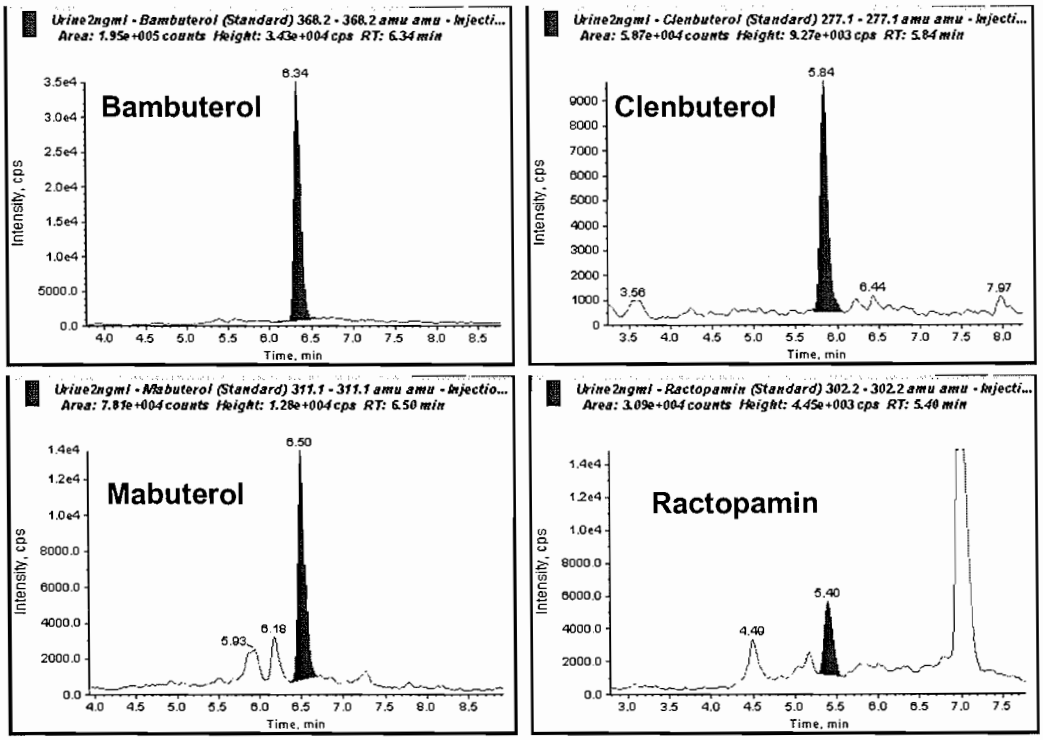

Figure 2. Representative extracted ion chromatograms of a spiked urine sample at a level of 2 ng/ml

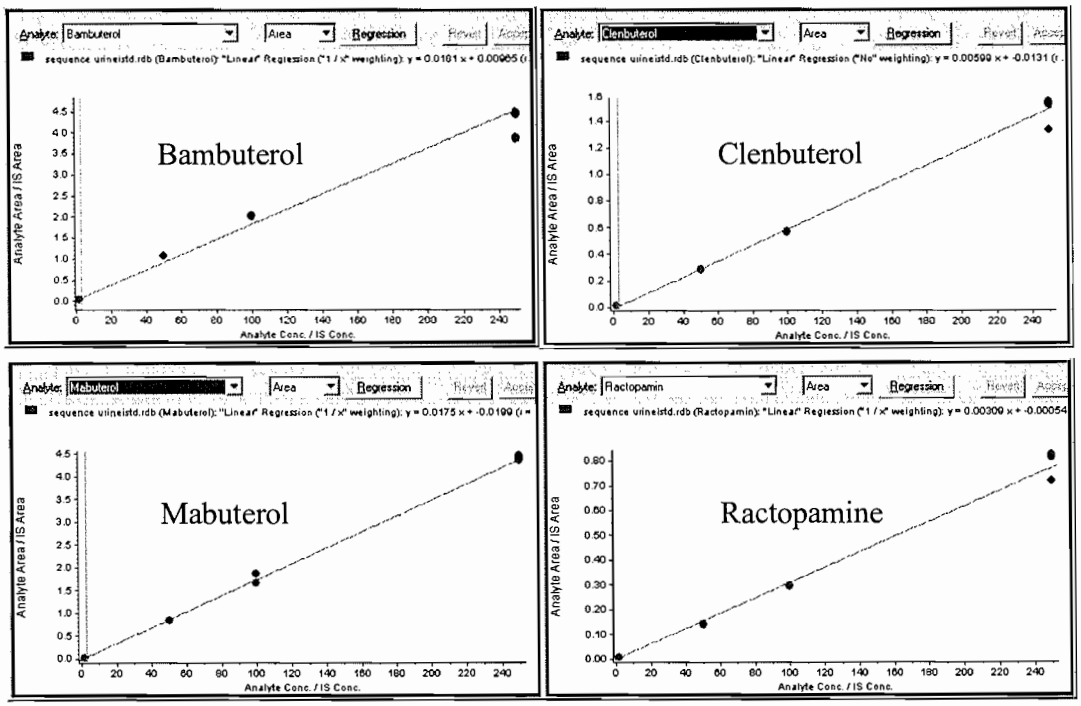

Figure 3. Calibration curves based on spiked urine samples (2 ng/ml – 250 ng/ml)

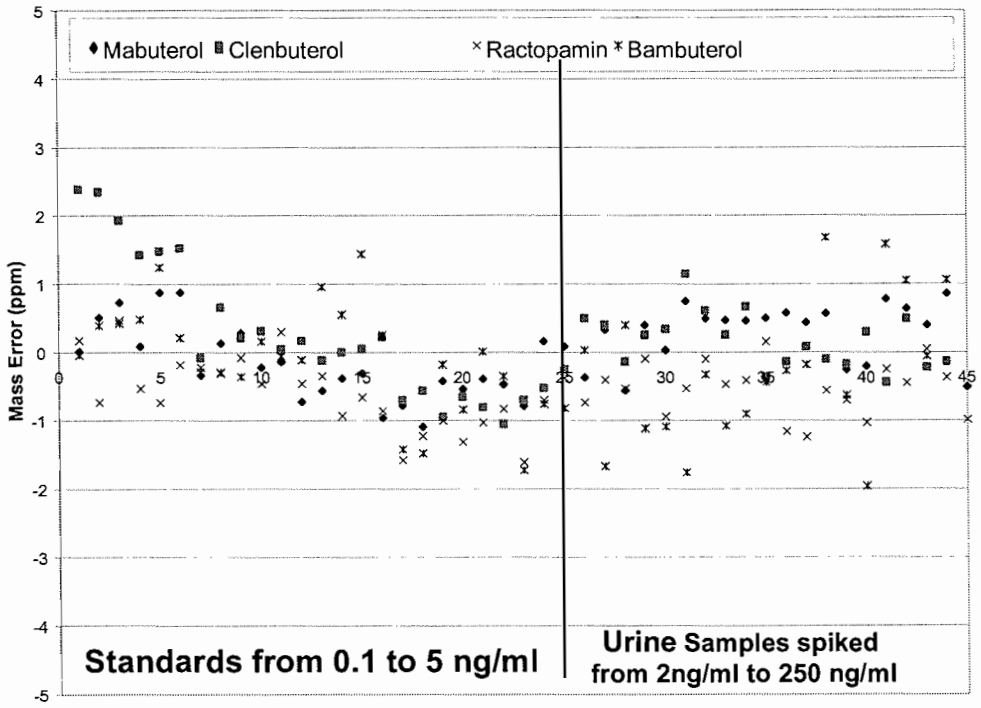

Figure 4. Mass accuracy on an 18 hour sequence for the investigated beta-2-agonists

Table 2: Calibration curve statistics

Bambuterol

Expected Conc.	No. Of Values	Low	High	Mean	Standard Deviation	%CV	Accuracy
2	5	1.59	1.67	1.65	0.035	2.104	82.40
50	5	58.11	58.56	58.36	0.185	0.318	116.72
100	5	110.50	111.93	111.27	0.640	0.575	111.27
250	5	211.46	247.25	232.40	17.934	7.717	92.96

Mabuterol

Expected Conc.	No. Of Values	Low	High	Mean	Standard Deviation	%CV	Accuracy
2	5	2.02	2.09	2.06	0.026	1.243	102.90
50	5	48.72	49.11	48.92	0.188	0.385	97.85
100	5	95.62	108.17	98.39	4.807	4.886	98.39
250	5	250.48	256.32	253.69	2.674	1.054	101.47

Clenbuterol

Expected Conc.	No. Of Values	Low	High	Mean	Standard Deviation	%CV	Accuracy
2	5	2.05	2.17	2.09	0.051	2.455	104.50
50	5	47.65	48.77	48.24	0.422	0.876	96.49
100	5	96.27	98.05	97.25	0.715	0.736	97.25
250	5	226.79	263.17	254.41	15.545	6.110	101.77

Ractopamin

Expected Conc.	No. Of Values	Low	High	Mean	Standard Deviation	%CV	Accuracy
2	5	2.14	2.24	2.21	0.040	1.832	110.51
50	5	44.27	45.78	45.09	0.705	1.563	90.18
100	5	95.13	96.59	95.71	0.632	0.661	95.71
250	5	232.60	267.43	258.99	14.825	5.724	103.60

Calibration Curves: Linear weighted 1/x

Literature:

Thevis, M. *et al.*: Liquid chromatography/electrospray ionization tandem mass spectrometric screening and confirmation methods for β_2-agonists in human or equine urine *J. Mass Spectrom.* 2003; **38**: 1197–1206