Correspondence case report

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Clenbuterol – regional food contamination a possible source for inadvertent doping in sports

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The misuse of the sympathomimetic and anabolic agent clenbuterol has been frequently reported in professional sport and in the livestock industry. In 2010, a team of athletes returned from competition in China and regular doping control samples were taken within the next two days. All urine samples contained low amounts (pg/ml) of clenbuterol, drawing the attention to a well-known problem: the possibility of an unintended clenbuterol intake with food. A warning that Chinese meat is possibly contaminated with prohibited substances according to international anti-doping regulations was also given by Chinese officials just before the Bejing Olympic Games in 2008.

To investigate if clenbuterol can be found in human urine, a study was initiated comprising 28 volunteers collecting urine samples after their return from China. For the quantification of clenbuterol at a low pg/ml level, a very sensitive and specific isotope dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay was developed using liquid/liquid re-extraction for clean-up with a limit of detection and quantification of 1 and 3 pg/ml, respectively. The method was validated demonstrating good precision (intra-day: 2.9–5.5 %; inter-day: 5.1–8.8%), accuracy (89.5–102.5%) and mean recovery (81.4%). Clenbuterol was detectable in 22 (79%) of the analyzed samples, indicating a general food contamination problem despite an official clenbuterol prohibition in China for livestock. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: clenbuterol; food contamination; sports drug testing; inadvertent doping; LC-MS/MS

Introduction

Cheating with clenbuterol has a long history in the livestock industry and competitive sports. Particularly, the growth-promoting effects of the sympathomimetic and anabolic agent such as muscle hypertrophy and fat degradation are the main reasons for its extensive misuse.[1,2] In the 1990s, the illegal administration of clenbuterol for livestock in the EU and the USA was described frequently.[3,4] However, current news reports indicate an ongoing misuse of the growth promoter as 'lean meat powder' for the livestock industry, especially in China and Mexico. In China, different cases of clenbuterol contaminated meat were reported between 2006 and 2010 with hundreds of poisoned people.[5–7] Interestingly, athletes were officially warned just before the Beijing Olympic Games in 2008 that Chinese meat is possibly contaminated with doping-relevant substances (pers. comm. Dr W. Kindermann, Chief Medical Doctor German Olympic Team, 2008). In 2008, an extensive clenbuterol misuse is reported for Mexico as well, with 76% positive findings of investigated bovine meat samples.[8,9] That the consumption of clenbuterol-contaminated meat can result in positive findings was described by Hemmersbach *et al.* demonstrating that clenbuterol was found in volunteers' urine with maximum concentrations of 850 pg/ml after consumption of meat from treated cattle, receiving two doses of 5 μg/kg/day clenbuterol up to one day before slaughtering.[10] Athletes visiting countries affected by clenbuterol misuse risk an unintended clenbuterol intake with food and consequently, inadvertent doping. Since clenbuterol belongs to the most frequently reported prohibited substances, according to the World Anti-Doping Agency's statistics with 116 findings in 2010, the present situation is difficult for effective doping control.[11] A differentiation between an unintended intake and a doping offense remains a challenging task. The present study was initiated based on a case in 2010, as a German team of athletes returning from China was tested; all urine samples contained trace amounts of clenbuterol in the low pg/ml range. To investigate, whether food contamination problems in China can have effects on doping control, the concentration of clenbuterol was determined in urine specimens provided by 28 volunteers after visiting China. To ensure a reliable quantification of clenbuterol in the lower pg/ml range a sensitive and specific assay was developed using an effective sample clean-up and analysis based on liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-(ESI)-MS/MS).

Experimental

Chemicals

Clenbuterol was obtained from National Measurement Institute (Sydney, Australia) and d_9-clenbuterol from LGC Standards GmbH (Wesel, Germany). Both were of analytical purity. Potassium

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hydroxide, sodium phosphate, and hydrochloric acid were purchased from Merck (Darmstadt, Germany) and acetic acid and ammonium acetate from Sigma (Steinheim, Germany). All reagents were of analytical grade. Acetonitrile (LC-MS grade) and t-butyl methyl ether (distilled before use) was supplied by VWR International GmbH (Darmstadt, Germany). Standard solutions and other aqueous solutions were prepared using deionised water (Sartorius Stedim Biotech S. A., Aubagne, France).

Study subjects

Urine samples were collected from 28 volunteers (13 female/15 male) travelling to China for different time-periods between 4 and 23 days. Nine out of the 28 subjects were permanent residents of China. Urine sampling was conducted for the travellers' group on the day of return to Germany and urine samples of the permanent residents were sent by courier. The visited areas and durations of stay of each volunteer are illustrated in Table 2. Since, the collection of negative control samples before the travel was possible only in 4 out of 28 specimens, an additional negative control group comprising 30 volunteers from Cologne, Germany, was investigated. An informed consent was obtained from each volunteer.

LC-MS/MS

The LC system consisted of an Agilent 1100 Series HPLC equipped with an Eclipse XDB-C_8 analytical column (4.6 × 150 mm, 3 µm particle size; Agilent, Waldbronn, Germany). The mobile phases were composed of 5 mM ammonium acetate containing 0.1% glacial acetic acid (pH = 3.5, mobile phase A) and acetonitrile (mobile phase B). A linear gradient at a flowrate of 0.8 ml/min was employed starting at 1% B, increasing to 60% B within 7 min and re-equilibrating at 1% B for 4 min before the next sample was injected. The total runtime was 11 min and the injection volume used was 50 µl.

Tandem mass spectrometry was carried out using a hybrid triple quadrupole/linear ion trap mass spectrometer (AB Sciex 5500 QTrap; Darmstadt, Germany) controlled by Analyst Software 1.5.1 (AB Sciex). Positive ionization mode heated electrospray was used with the following conditions: ionspray voltage +5500 V, ion source temperature 450 °C, nitrogen was used as curtain, nebulizer, auxiliary and collision gas (3.0×10^{-3} Pa) delivered from a nitrogen generator (CMC Instruments, Eschborn, Germany). Clenbuterol and d_9-clenbuterol (IS) were detected utilizing multiple reaction monitoring (MRM) of diagnostic ion transitions at dwell times of 80 msec (Table 1). Samples were quantified by isotope dilution using an external calibration.

Sample preparation

Samples were prepared based on a previously published protocol modified for high sensitivity and reliable quantification of clenbuterol in the low pg/ml range.[12]

To aliquots of human urine (5 ml) 0.3 ng of IS (d_9-clenbuterol) and 50 µl of potassium hydroxide (5 M, pH 12.0) were added. Liquid-liquid extraction was performed using 6 ml of t-butyl methyl ether (TBME). After shaking (10 min) and centrifugation (1250 *g*, 5 min) the upper ether layer was separated. For re-extraction 400 µl of hydrochloric acid (0.06 M) were used and after subsequent shaking and centrifugation (see above) the ether layer was discarded. Final extracts were stabilized adding 50 µl of phosphate buffer (0.8 M, pH 7) and injected into the LC-MS/MS system.

Table 1. Summary of MS and validation parameters

Precursor Ion	Product Ion I	CE I	Product Ion II	CE II	Product Ion III	CE III	LOD (S/N > 3)	LOQ (S/N > 9)	Linearity	Precision		Accuracy	Recovery
(*m/z*)	(*m/z*)	(eV)	(*m/z*)	(eV)	(*m/z*)	(eV)	(pg/ml)	(pg/ml)		intra-day (n = 6/6/6)	inter-day (n = 18/18/18)	(n = 6/6/6)	(n = 6/6)
277	168	41	132	37	140	67	1	3	$y = 0.0054 \cdot x - 0.0036$ $R^2 = 0.997$	2.9 - 5.5 %	5.1 - 8.8 %	89.5 - 102.5 %	81.4 %

Validation

For validation, the parameters specificity, ion suppression, linearity, intra- and inter-day precision, accuracy, recovery and limit of detection (LOD), and quantification (LOQ) were determined according to the guidelines of the International Conference on Harmonisation (ICH) and WADA.[13,14] All calibration samples were prepared and analyzed as described above.

Specificity

Specificity was tested by analyzing six different blank urine samples collected from healthy volunteers (3 male/ 3 female) to test for interfering signals in the selected MRM chromatograms at expected retention times of the analytes.

Linearity

Calibration curves ($n = 7$) for clenbuterol were generated using aliquots of a blank urine sample spiked at concentration of 3, 5, 10, 25, 50, 75, and 100 pg/ml, respectively.

Ion suppression/ion enhancement

Ion suppression or enhancement effects were investigated by analyzing six different blank urine samples via post-column continuous infusion of pure clenbuterol reference solution (1 ng/ml, 10 µl/min) and observation of the ESI response at the retention time of clenbuterol.[15]

Precision and accuracy

Intra-day precision and accuracy were determined using six replicates of spiked urine samples at the concentration levels of 5, 50, and 100 pg/ml (QC_{low}, QC_{medium}, QC_{high}). To establish the inter-day precision the same samples were prepared and analyzed on three consecutive days ($n = 6 + 6 + 6$). The precision of the method was determined by calculation of the coefficient of variation (CV) of the area ratio of the quantifier ion transition *m/z* 277–168 for clenbuterol and *m/z* 286–204 for d_9-clenbuterol. Accuracy was investigated calculating the concentrations of the respective aliquots using an external calibration curve.

Recovery

The recovery of the assay was determined using two sets of different blank urine samples (n = 6) fortified with clenbuterol reference compound at a concentration level of 50 pg/ml before and after sample preparation. The IS d_9-clenbuterol was spiked to the final extract of both sets of samples. Recovery was calculated by comparison of the mean relative peak areas in the different sets of samples.

Limit of detection and quantification

The LOD and the LOQ were defined as the lowest concentrations of the analyte in a sample that give signal-to-noise ratios of 3:1 and 9:1, respectively. Six aliquots of blank urine samples were analyzed to establish the noise intensity, six aliquots were spiked at 1 pg/ml and another 6 at 3 pg/ml to estimate the LOD and LOQ.

Results and discussion

Validation

The assay was validated regarding the parameters specificity, ion suppression, linearity, intra- and inter-day precision, accuracy, recovery, and LOD and LOQ. Good specificity was demonstrated with the analysis of six different blank urine samples generating no interfering signals in the selected MRM chromatograms for clenbuterol (Figure 1) and no ion suppression or enhancement effects were observed. The assay demonstrated a linear correlation between analyte concentration and response within the concentration range (3–75 pg/ml) with $y = 0.0054\ x$ - 0.0036 and correlation coefficients (R^2) above 0.99. As summarized in Table 1, calculated CVs for intra-day precision ranged from 2.9 to 5.5% and for inter-day from 5.1 to 8.8% at three different concentrations. Accuracy was determined by comparison of theoretical and calculated concentrations at a low, medium, and high concentration level (5, 50, and 100 pg/ml) with calculated relative recoveries ranging from 89.5 to 102.5% (mean 95.3 %) for clenbuterol. The absolute recovery was calculated for clenbuterol as described above with 81.4%. High sensitivity was achieved with a determined LOD of 1 pg/ml (S/N = 3) and a LOQ of 3 pg/ml (S/N = 9) using three diagnostic ion transitions (Table 1).

Studied subjects

A total of 28 volunteers (13 female, 15 male) were investigated consisting of 19 travellers visiting China and 9 permanent residents of different regions of China (Table 2). Urine sampling was conducted for the travellers group immediately after their return to Germany to minimize the elimination of clenbuterol from the body. The study was designed to simulate common athletes' doping control samples. Thus, no restrictions regarding diet were followed. Only vegetarian nutrition and the use of clenbuterol containing drugs were questioned. The investigated specimens (n = 28) demonstrated 22 positive (79%) and 6 (21%) negative findings (< LOD) for clenbuterol. One of the negative samples belonged to a vegetarian volunteer. The concentration of clenbuterol in 14 out of the 22 positive samples ranged between 3.1 and 50.5 pg/mL, while the concentration of 8 samples ranged between 1 (LOD) and 3 (LOQ) pg/mL. Generally, no connection was observed regarding the visited area and clenbuterol levels, except for Hong Kong were a tendency towards lower concentrations (<LOD n = 3; < LOQ n = 3) was observed. It was possible only for four volunteers to provide negative control samples before travelling to China. All four urine samples were tested negative. Taking into account the low number of negative control samples for the travellers, an additional negative control group (n = 30, Cologne, Germany) was investigated providing no findings for clenbuterol. Generally, the risk of an unintended clenbuterol intake with food in Germany or the EU is supposed to be very low, as the Federal Office for Consumer Protection and Food Safety of Germany reported no findings for clenbuterol misuse in Germany in 2009.[16] In addition, the analysis of thousands of official doping control samples collected by the German doping control authorities indicate no clenbuterol food contamination problem in Germany.

Only few data are available on the urinary excretion of clenbuterol in humans. Yamamoto *et al.* investigated the cumulative urinary excretion of clenbuterol after a single dose of 20 µg.[17] During the first 24 h, a mean cumulative urinary excretion of 15.1% was reported. Considering a common daily urine volume of two litres, a corresponding theoretic urinary concentration of 1.5 ng/ml can be calculated. According to literature the concentrations in meat (muscle) after administration of typical doses of 20 µg/kg clenbuterol to pigs yielded maximum

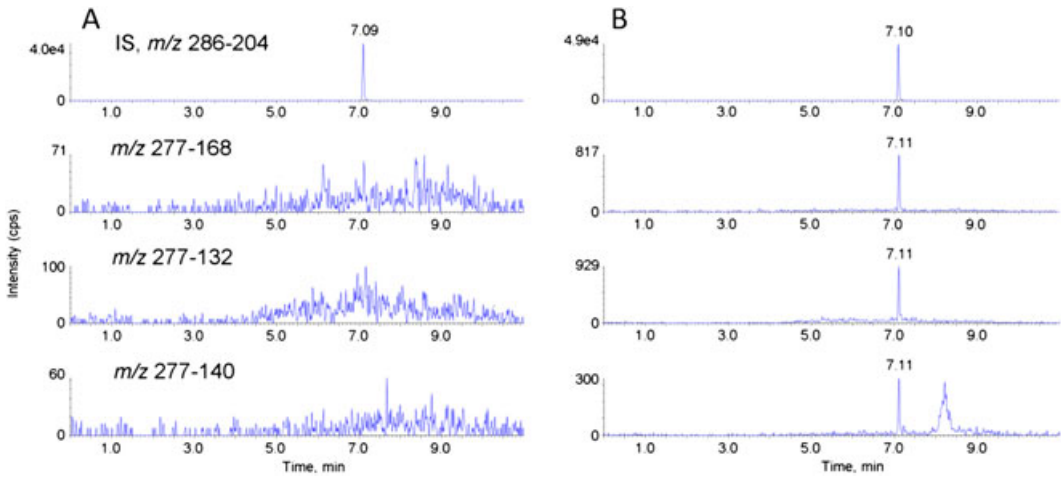

Figure 1. MRM chromatograms of a blank sample (A), and a volunteers' urine sample at a clenbuterol concentration of 3.1 pg/mL, representing the LOQ of the method (B).

Table 2. Summary of investigated volunteers and urinary clenbuterol concentration

Volunteer	Sex	Duration of stay	Visited area	Conc. (pg/ml)
V008	Female	11.11.10-17.11.10	Beijing, Guiein, Shanghai	ND[a]
V014	Female	11.11.10-02.12.10	Hongkong	ND[a]
V015	Male	11.11.10-02.12.10	Hongkong	ND[a]
V020	Female	Permanent resident of Shanghai	Shanghai	ND[a]
V022	Male	Permanent resident of Shanghai	Shanghai	ND[a]
V024	Male	Permanent resident of Hongkong	Hongkong	ND[a]
V013	Male	13.11.10-21.11.10	Beijing, Chongqing, Shanghai	1-3[b]
V026	Female	Permanent resident of Hongkong	Hongkong	1-3[b]
V027	Male	Permanent resident of Hongkong	Hongkong	1-3[b]
V021	Male	Permanent resident of Shanghai	Shanghai	1-3[b]
V023	Female	15.11.10-05.12.10	Shanghai	1-3[b]
V025	Male	Permanent resident of Hongkong	Hongkong	1-3[b]
V002	Male	20.10.10–30.10.10	Beijing	1-3[b]
V016	Female	03.12.10-09.12.10	Beijing	1-3[b]
V011	Female	17.11.10-27.11.10	Shanghai	3.1
V007	Female	07.11.10-13.11.10	Shanghai, Hangzhou, Shanghai	3.9
V028	Male	07.01.11-15.01.11	Beijing	4.9
V017	Female	13.12.10-17.12.10	Beijing	5.3
V012	Male	17.11.10-27.11.10	Shanghai	5.4
V004	Male	20.10.10–30.10.10	Beijing	6.2
V006	Female	07.11.10-14.11.10	Shanghai, Wuxi	6.3
V009	Male	13.11.10-21.11.10	Beijing, Chongqing, Shanghai	7.1
V019	Female	Permanent resident of Nanchang	Nanchang, Jiang Xi Province	7.1
V010	Male	12.11.10-20.11.10	Shanghai, Nanjing, Wuxi, Qingdao	10.7
V005	Female	19.10.10-01.11.10	Hangzhou, Shanghai	22.4
V003	Male	20.10.10–30.10.10	Beijing	22.6
V001	Male	08.10.10–20.10.10	Hainan	25.0
V018	Female	Permanent resident of Nanchang	Nanchang, Jiang Xi Province	50.5

[a]ND: Not detectable (< LOD)
[b]> LOD (1 pg/ml) < LOQ (3 pg/ml)

concentrations of 4.4 to 6.1 μg/kg.[8,18] Assuming a consumption of 300 g of contaminated meat (muscle), these amounts would result in an intake of 1.3–1.9 μg of clenbuterol, which is approx. one-tenth of a typical therapeutic single dose of 20 μg used in man. Based on this data, maximum urinary concentrations are supposed to range between 100 and 150 pg/ml. However, the misuse of clenbuterol for livestock is not controlled and applied doses are expected to be much higher, as demonstrated by the large number of clenbuterol poisonings.[5–7] The results obtained within the present study with maximum concentrations of 50.5 pg/ml are difficult to compare, since the amount of clenbuterol ingested was unknown.

Conclusion

We have presented a very sensitive and specific assay for the quantification of urinary clenbuterol in the pg/ml range. The data collected within this study and from literature demonstrated that food contamination with clenbuterol is a serious problem in China and Mexico, despite its official prohibition for livestock. Thus, athletes visiting those areas take a risk of an unintended clenbuterol intake with food and consequently, inadvertent doping. Additionally, and more serious a clenbuterol food contamination is a major problem of public health. For doping control the situation is very unsatisfying, since a differentiation between an unintended intake and cheating stays difficult.

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