M. Kohler[1], A. Thomas[1], K. Walpurgis[1], L. Horta[2], W. Schänzer[1], M. Thevis[1]

Detection of proteases and their proteolysis and autolysis products in urine by LC-MS

[1]Institute of Biochemistry / Center for Preventive Doping Research, German Sport University Cologne, Germany

[2]Laboratorio de Analises de Dopagem, Instituto do Desporto de Portugal, Lisboa, Portugal

Abstract

The use of peptide hormones for performance enhancement is documented by positive doping control samples with e.g. human chorionic gonadotropin or erythropoietin and by confession of athletes or confiscation of peptide hormone products. With the development of doping control methods, dopers start searching for manipulation and masking strategies to circumvent the detection of prohibited substances in urine samples. One way to eliminate peptide hormones from doping control samples is the use of proteases that degrade all proteins in urine and beside the proteolytic activity usually also autolyse. A method was established and validated to detect proteases and their degradation products in urine by SDS-PAGE as a screening procedure and LC-MS(/MS) for confirmation and for identifying a specific protease in the sample. Two doping control urine samples were shown to be positive for bacillolysin and four seized products for bacillolysin, subtilisin and aspergillopepsin.

Introduction

Proteases are enzymes that are capable of circumventing the detection of peptide hormones from urine samples[1-6] and the addition to urine is therefore prohibited by the World Anti-Doping Agency (WADA) in the category "chemical and physical manipulation"[7]. Proteases degrade proteins and different proteolytic enzymes have a different specificity in fragmenting proteins. Additionally, proteases with varying specificity and efficiency also have a different capability to autolyse and produce small peptides originating from the protease itself.

Proteases are of vegetable or animal origin and in the past 20 years a large variety of xenobiotic proteases with specific properties in respect to activity at different pH and temperature ranges, different specificity and different efficiency in proteolyis and autolysis have been recombinantly produced. Washing powder is one of the usual protease products which demonstrates that products with proteases can easily be obtained by cheating

sportsmen. Washing powder often contains subtilisin as a protease which is very efficient in degrading proteins. Therefore, a method for the detection of proteases needs to be as flexible as possible to cover the detection of various fragments of different proteases. Also for a certain protease the degradation products largely depend on the storage conditions of the samples as well as e.g. salt and protein concentration. In contrast, human endogenous proteases are rare in urine and mass spectrometric detection was achieved only when large amounts (~ 200 mL) of urine were prepared for analysis. Trypsinogen, chymotrypsinogen and collagenase were identified in urine earlier[8, 9] but did not seem to have proteolytic activity as intact urinary proteins were determined.

The problem of erythropoietin (EPO) detection after manipulation with proteases was recognized and investigated earlier, and the detection of urine manipulation was performed by trypsin digestion of the intact proteases (e.g., chymotrypsin, papain and trypsin) after separation on SDS-gels[10, 11]. The aim of this work was to develop a method that enables the detection of degradation products of the proteases in urine by LC-MS(/MS) even if no intact protease is visible on SDS-PAGE. Doping control analysis requires a distinct identification of the protease misused, which means that the SDS-PAGE alone and the disappearance of a normal protein pattern is not adequate to report an adverse analytical finding. Therefore, the earlier described SDS-PAGE method[10] was applied to screen urine samples for unusual protein patterns, and a LC-MS/MS-based assay was developed to confirm the presence of protease degradation products in doping control specimens. The first two positive samples proved the successful application of the method.[12, 13]

Materials and Methods

Proteases (subtilisin A (P5380), papain (P4762), trypsin (T4665)), as well as dithiothreitol (DTT) and trifluoroacetic acid (TFA, both analytical grade) were purchased from Sigma (Deisendorf, Germany). Bacillolysin (P1236) from Sigma (Deisendorf, Germany) was obtained as a protease solution from *Bacillus amyloliquefaciens* with a lot related activity of 0.90 U/g. Solid phase extraction cartridges OASIS HLB (60 mg, 3 cm^3) were bought from Waters (Eschborn, Germany) and Amicon centrifugal filters (cut-off 10 kDa) were obtained from Millipore (Schwalbach, Germany). 12% Bis-Tris gels, 3-(*N*-morpholino)propanesulfonic acid (MOPS) running buffer and lithium dodecyl sulphate (LDS) sample buffer were from Invitrogen (Karlsruhe, Germany) and Coomassie Blue gel stain from Pierce (Rockford, IL**,** USA). Methanol, acetonitrile and acetic acid (all analytical grade) were from Merck (Darmstadt, Germany). All aqueous buffers and solutions were prepared in MilliQ water.

Urine samples

All urine specimens for method development and validation were spontaneous urine samples obtained from healthy male and female volunteers and were stored at 4°C until analysis. Urine samples were heated to 37°C prior to addition of the respective protease with subsequent cooling to room temperature to simulate authentic conditions for urine adulteration by cheating sportsmen.

Doping control urine samples were stored at 4°C until they were suspicious for proteases (2-3 weeks) and were then kept frozen.

SDS-PAGE of urine samples

Screening for unusual protein patterns in urine samples was accomplished by concentrating 4 mL of urine in a centrifugal filter (10 kDa cut-off, 25 min, 4000 g) to approximately 50 µL. A volume of 20 µL of the retentate was mixed with 7 µL of LDS sample buffer (4x) and 3 µL of DTT and heated for 10 min at 70 °C. After SDS-PAGE (12% Bis-Tris gel, MOPS running buffer, 125 V) gels were stained with Coomassie Blue.

Liquid chromatography – (tandem) mass spectrometry

Sample preparation was performed by solid phase extraction (SPE) with 1 mL of urine added to an OASIS HLB (60 mg, 3 cm^3) SPE cartridge that was preconditioned consecutively with 2 mL of acetonitrile and 2 mL of water. The cartridge was washed with 2 mL of water and elution of analytes was accomplished with a mixture of water/acetonitrile (1.4 mL, 20/80, v:v). The eluate was evaporated to dryness in a vacuum centrifuge prior to reconstitution with 100 µL of acetic acid (2%). Liquid chromatography was performed on an Agilent (Böblingen, Germany) 1100 HPLC equipped with a Zorbax 300 SB trapping column (5 x 0.3 mm, particle size 3.5 µm, 50 µL/min) and a Zorbax 300 SB-C18 analytical column (50 x 0.3 mm, particle size 3.5 µm, 10 µL/min, both Agilent). Solvent A was 0.1 % acetic acid containing 0.01 % of trifluoroacetic acid, and solvent B was composed of 80 % acetonitrile with 0.1 % of acetic acid and 0.01 % of trifluoroacetic acid. The following gradient was used: 0-2 min 95 % A, 2-25 min 60 % A, 25-34 min 3 % A, 34-44 min 3 % A, re-equilibration 16 min 95 % A. Mass spectrometric analysis was done on a high resolution/high accuracy instrument (Thermo LTQ Orbitrap, Bremen, Germany) with electrospray ionization in positive mode and an ionisation voltage of 3.5 kV. The resolution was set to 30,000 (FWHM), and the collision energy for automated data-dependent MS/MS experiments, that were performed using the linear ion trap

mass analyzer, was 35 % (arbitrary units, Xcalibur software version 2.0, Thermo Fisher Scientific, 2006). Calibration of the instrument was performed prior to measurement using the manufacturer's calibration mixture to ensure accurate mass determination.

Data analysis

For the evaluation of LC-MS/MS data, Proteome Discoverer including the Swissprot/Uniprot database (2008, Thermo, Bremen, Germany) was used. The search was not restricted to a certain enzyme and the database was completely searched without predefinition of species. The identification of proteins was considered successful if at least two peptides were detected and the peptide masses did not deviate more than 10 ppm from the theoretical values. Mass tolerance for product ions was set to 0.8 Dalton.

Standard solutions

Aqueous protease standard solutions (1 mg/mL) were prepared and stored at -20°C. They were used at most three times to avoid a loss of activity due to freeze and thaw cycles. The bacillolysin stock solution was stored at 2-8°C and used without further dilution.

Validation

The method is intended to be used as an analytical tool to detect the misuse of proteases for urine alteration. Therefore a validation for qualitative purposes was performed considering specificity, working range, repeatability, robustness, stability and limit of detection.[12]

Results and Discussion

Manipulation of urinary specimens in sports drug testing with proteolytic enzymes provides a simple and highly effective tool to disable common peptide as well as protein analysis. Thus, an analytical approach for the direct identification of exogenous proteases and their degradation products in urine provides a helpful progress for doping control. To enable the detection of proteases that are not visible as characteristic bands in SDS-PAGE, which was described and used for doping control purposes earlier[10], a LC-MS/MS-based approach was developed.

Figure 1 shows a SDS-gel with a blank urine concentrate without and with the addition of different proteases. The protein pattern demonstrates the different efficiency of the proteases and the way that confirmatory analysis is dependent on the protein pattern on the gel which is also indicated in Figure 1. If a potential protease band is visible the confirmation is performed

as described before[10]. If no suspicious band is visible, but the protein pattern is suspicious, a confirmation by solid phase extraction (SPE) and LC-MS(/MS) is undertaken.
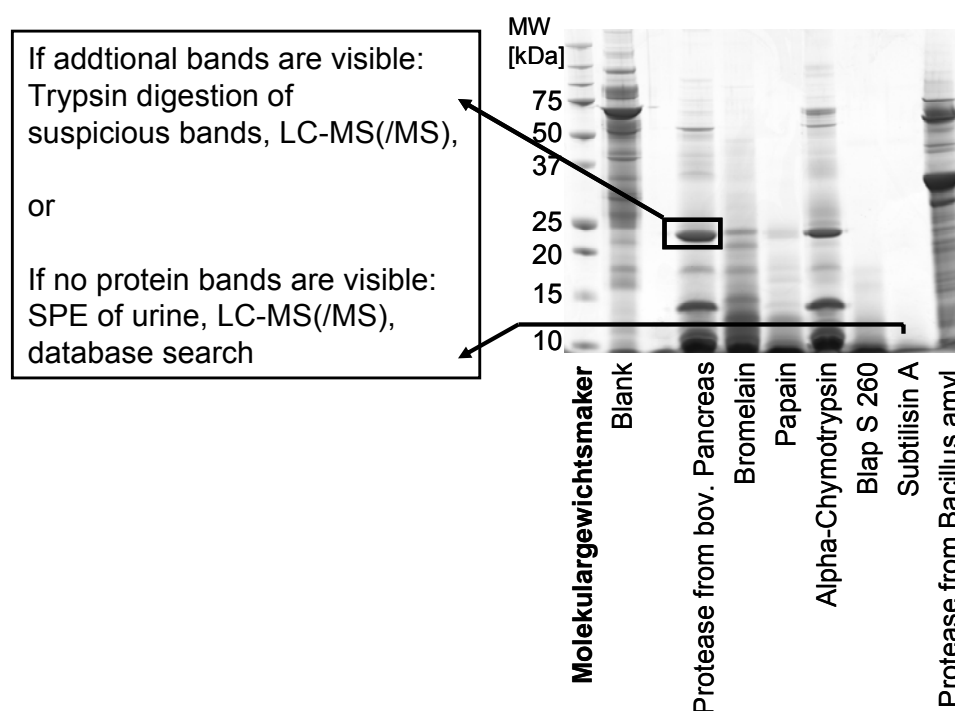

Figure 1: SDS-PAGE of a blank urine sample and blank samples incubated with different proteases.

While e.g. chymotrypsin or bacillolysin produce specific protease bands after a comparably short incubation time (30 min room temperature), subtilisin or papain are very efficient in proteolysis and autolysis and the samples rarely show any protein. Also in the case of proteases such as chymotrypin or bacillolysin the protease concentrations may be too low to produce a visible band on the gel but may nevertheless be detected after SPE and LC-MS(/MS). Additionally, degradation products can be detected and then attributed to the corresponding protease or protein.

After SPE and LC-MS(/MS) the evaluation of the raw data needs to be done by a database search because the problem is much too complex to analyze the data manually. If no band is detected, no indication as to which protease could be present in the sample is available. Furthermore, the use of non-specific proteases or combinations of proteases will produce unpredictable protein fragments and proteases may be from different origin. Therefore, the LC-MS(/MS) run includes data-dependent MS/MS experiments meaning that the three most intensive peptides (signals with charge > 1) in a scan are fragmented and MS/MS spectra recorded. Mass spectra and MS/MS spectra are then evaluated by the database

(Swissprot/Uniprot) and compared to theoretical spectra from all species with any possible cleavage site. The database evaluates the hits and lists the identified proteins and their peptides. In this method – for a successful confirmation – at least 2 peptides need to be detected with a mass error of the monoisotopic peptide < 5 ppm, the sequence coverage needs to be ≥ 10% and the retention time should not deviate more than 2 % from a urinary reference standard.

As an example, two urine samples and four powders were found to contain different proteases.

Figure 2 shows the SDS-PAGE screening as well as an activity test for the urine samples. Samples 1 and 2 do not show a normal protein pattern when compared to the blank sample which makes them suspicious that they contain proteases. They show very little medium or high molecular weight proteins but a higher number of smaller proteins or protein fragments. Additionally both samples exhibited protease activity and added proteins (growth hormone and myoglobin) were degraded within 30 min at 37 °C. The four confiscated powders did not show any intact protein on the SDS-gel but some signals of < 10 kDa, which may be degraded proteins.
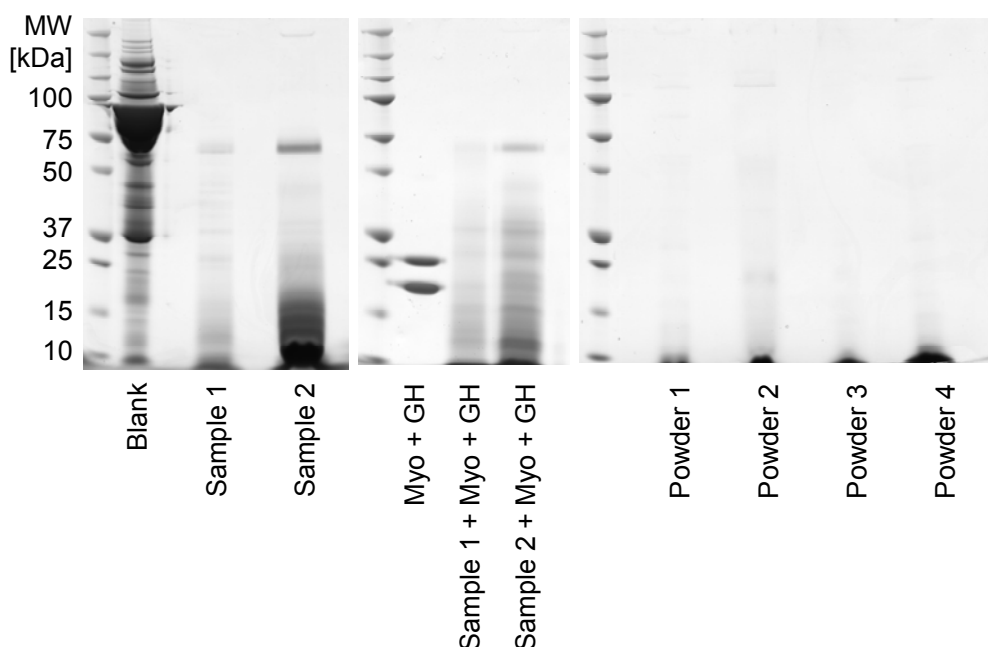

Figure 2: Gel on the right site: Blank sample and two urine samples not showing a typical protein pattern. Gel in the middle: Activity test, Myo=myoglobin and GH=growth hormone were added to the urine samples but are not visible on the gel due to protease activity. Gel on the left: SDS-PAGE of four confiscated powders that do not show intact proteins

After the screening test showed both samples and the four powders to be suspicious containing proteases, by SDS-PAGE and an activity test followed by SDS-PAGE was also suspicious, the confirmation and identification of the protease was done by LC-MS(/MS).

In both of the two samples bacillolysin from *Bacillus amyloliquefaciens* was identified with a sequence coverage of 17 and 33% and 5 and 15 peptides respectively. Additionally a urinary bacillolysin standard showed two peptides that were identical to those detected in the samples and had a retention time shift of < 2% (see also Figure 4). Figure 3 demonstrates the results of the database search for sample 2 listing the peptides belonging to bacillolysin, their masses and mass errors as well as the retention time as detected in the LC-MS/MS run.

Sample 2 - Protein and Peptide database report
Bacillolysin(EC 3.4.24.28, P06832)

RT	Peptide	MH+	error (ppm)	z
12.97	H.AATTGTGTTLKGK.T	1206,67	2,55	2
13.25	A.TTGTGTTLKGK.T	1064,59	1,07	2
13.37	T.KIGVNKAEQ.I	986,56	1,73	2
13.41	S.STFKDAKAA.L	938,49	2,23	2
13.91	K.FNRNSYDNKGGK.I	1399,67	0,83	3
14.64	S.SQRAAVDAHYN.L	1231,58	1,29	3
15.66	N.SYDNKGGKIVS.S	1167,60	0,70	2
16.24	T.ITKIGVNK.A	872,56	1,75	2
16.70	M.THGVTQETANLN.Y	1284,62	1,98	2
16.81	T.ITKIGVNKAEQ.I	1200,69	1,39	2
18.59	Y.LTPSSTFKDAKAA.L	1336,71	2,31	2
18.76	K.IVSSVHYGSRY.N	1267,64	2,05	2
21.40	L.SKPTGTQIITY.D	1208,65	1,02	2
21.76	R.AAVDAHYNLGKVY.D	1420,72	2,43	2

Sequence coverage: 33 %

Figure 3: Peptides found in sample 2 proving the presence of bacillolysin in the urine sample

All four powders contained substilisin and bacillolysin from *Bacillus amyloliquefaciens* confirmed by high sequence coverages for bacillolysin (P06832, 41-78%) and subtilisin (P00782, 30-65%). Additionally, in three out of the four powders (powders 2-4) aspergillopepsin A from *Aspergillus awamori* (P17946) was detected and another Subtilisin produced in *Bacillus subtilis* (P35835) was identified and fulfilled the identification criteria (sequence coverage 12-26%, 4-11 peptides). Interestingly, the combination of the three proteases comprise the whole pH range: an alkaline protease (subtilisin), a neutral protease

(bacillolysin) and an acidic enzyme (aspergillopepsin), which appears like an intended mixture to cover the degradation of all kinds of urinary proteins
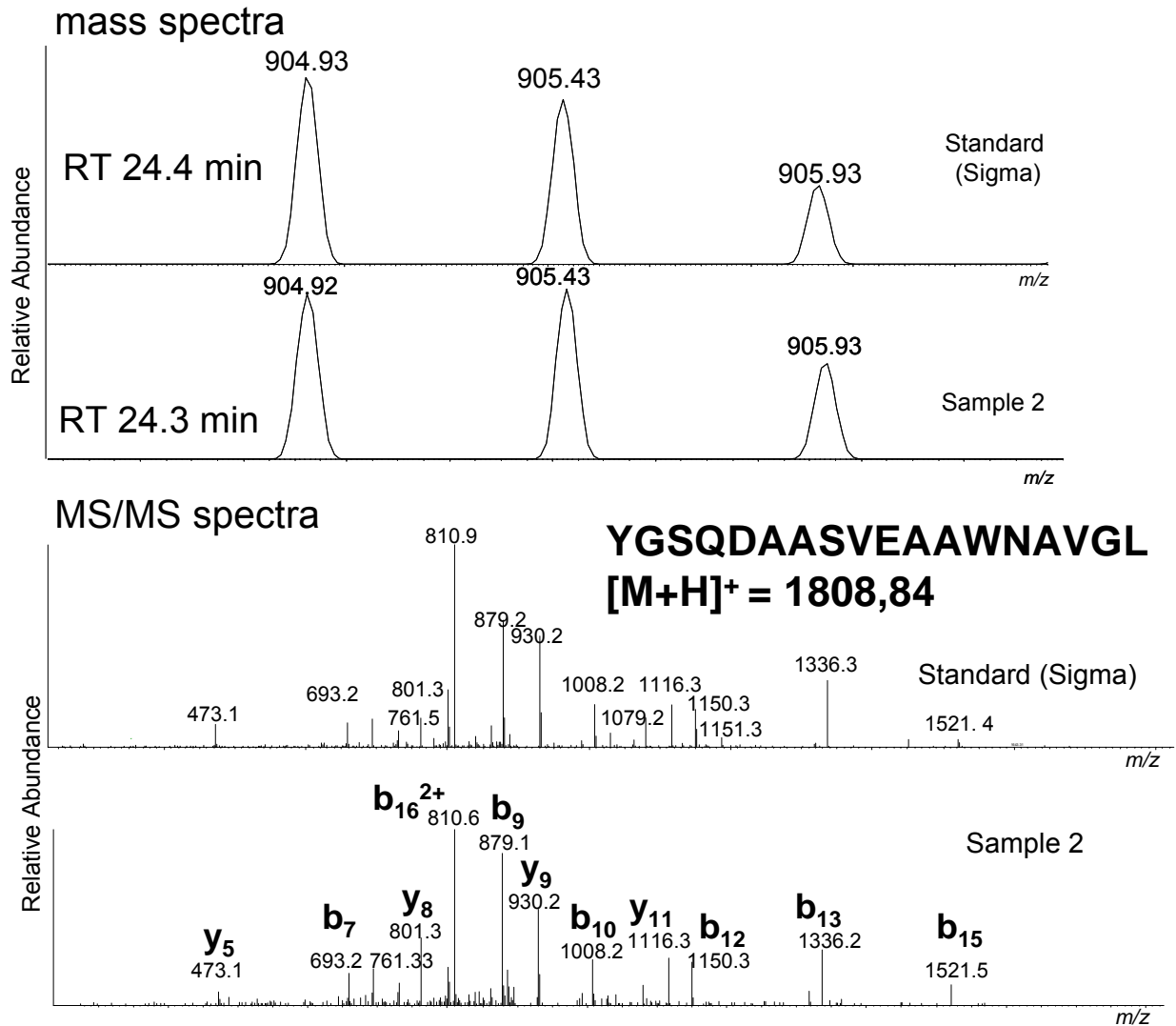

Figure 4: Example of mass spectrum and MS/MS spectrum of a peptide identified in sample 2 as well as the standard. The retention times are nearly identical as indicated in the mass spectrum (top two traces).

Figure 4 demonstrates the way the peptides and corresponding proteins were identified. The upper part of the Figure shows mass spectra of a peptide belonging to Bacillolysin that was found in sample 2 as well as in the bacillolysin standard. In the lower part, tandem mass spectra of both measurements are presented. The mass spectrum as well as the MS/MS spectra and the retention time of sample and standard fit perfectly with each other.

Concluding Remarks

The method developed here improves the detection method described earlier by enabling the detection of protease degradation products from urine samples, when no intact protease is visible on SDS-PAGE. This allows the detection of proteases such as Subtilisin that autolyse very quickly and also facilitates detection when protease cocktails are used. The more experience that can be gained with exogenous proteases in urine samples the longer the list of detected proteases will become and the method easily allows the implementation and detection of new or other proteases or mixtures.

Furthermore, selection of samples that should be tested for proteases could be done by choosing samples from EPO analysis, where no EPO is detected in the doping control analysis. Those could then be applied to SDS-PAGE and, if suspicious, to LC-MS/MS analysis.

Acknowledgements

References

1. Lasne F, Martin L, Crepin N, de Ceaurriz J. (2002) Detection of isoelectric profiles of erythropoietin in urine: differentiation of natural and administered recombinant hormones. *Anal Biochem.***311**, 119-126.
2. Thomas A, Thevis M, Delahaut P, Bosseloir A, Schanzer W. (2007) Mass spectrometric identification of degradation products of insulin and its long-acting analogues in human urine for doping control purposes. *Anal Chem.***79**, 2518-2524.
3. Thevis M, Thomas A, Delahaut P, Bosseloir A, Schanzer W. (2006) Doping control analysis of intact rapid-acting insulin analogues in human urine by liquid chromatography-tandem mass spectrometry. *Anal Chem.***78**, 1897-1903.
4. Thomas A, Kohler M, Schanzer W, Kamber M, Delahaut P, Thevis M. (2009) Determination of Synacthen in urine for sports drug testing by means of nano-ultra-performance liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom.***23**, 2669-2674.
5. Thomas A, Geyer H, Kamber M, Schanzer W, Thevis M. (2008) Mass spectrometric determination of gonadotrophin-releasing hormone (GnRH) in human urine for doping control purposes by means of LC-ESI-MS/MS. *J Mass Spectrom.***43**, 908-915.
6. Thomas A, Schänzer W, Delahaut P, Thevis M. (2009) Sensitive and fast identification of urinary human, synthetic and animal insulin by means of nano-UPLC coupled with high-resolution/high-accuracy mass spectrometry. *Drug Testing and Analysis.***1**, 219-227.
7. WADA (2009) The 2009 prohibited list: http://www.wada-ama.org/rtecontent/ document/2009_List_En.pdf, access: 01. February 2009.

8. Oh J, Pyo JH, Jo EH, Hwang SI, Kang SC, Jung JH, Park EK, Kim SY, Choi JY, Lim J. (2004) Establishment of a near-standard two-dimensional human urine proteomic map. *Proteomics.***4**, 3485-3497.

9. Spahr CS, Davis MT, McGinley MD, Robinson JH, Bures EJ, Beierle J, Mort J, Courchesne PL, Chen K, Wahl RC, Yu W, Luethy R, Patterson SD. (2001) Towards defining the urinary proteome using liquid chromatography-tandem mass spectrometry. I. Profiling an unfractionated tryptic digest. *Proteomics.***1**, 93-107.

10. Thevis M, Maurer J, Kohler M, Geyer H, Schanzer W. (2007) Proteases in doping control analysis. *Int J Sports Med.***28**, 545-549.

11. Lamon S, Robinson N, Sottas PE, Henry H, Kamber M, Mangin P, Saugy M. (2007) Possible origins of undetectable EPO in urine samples. *Clin Chim Acta.***385**, 61-66.

12. Thomas A, Kohler M, Walpurgis K, Schänzer W, Thevis M. (2009) Proteolysis and autolysis of proteases and the detection of degradation products in doping control. *Drug Testing and Analysis.***1**, 81-86.

13. Kohler M, Thomas A, Geyer H, Horta L, Schänzer W, Thevis M. (2009) Detection of the protease Bacillolysin in doping-control urine samples. *Drug Testing and Analysis.***1**, 143-145.