Research Article

Drug Testing and Analysis

Received: 21 June 2009 Revised: 21 August 2009 Accepted: 4 October 2009 Published online in Wiley Interscience:

(www.drugtestinganalysis.com) DOI 10.1002/dta.80

Comparison of the urinary protein patterns of athletes by 2D-gel electrophoresis and mass spectrometry – a pilot study

Maxie Kohler,[a] Stefan Franz,[b] Axel Regeniter,[c] Anna Ikonen,[a] Katja Walpurgis,[a] Andreas Thomas,[a] Wilhelm Schänzer,[a] and Mario Thevis[a]*

Urinary proteins and exercise-induced proteinuria have been the subject of much research. Proteinuria has been studied in depth after different running and cycling intensities and durations and the different mechanisms of glomerular filtration and tubular dysfunction have been elucidated. The present study was carried out to compare urinary protein profiles of athletes in different sport categories (endurance sport, team sport, strength sport). Doping-control urine samples obtained from in-competition testing and specimens derived from a control group were analysed by means of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and significantly deviating protein spots were enzymatically hydrolysed and identified by nanoflow liquid chromatography-orbitrap mass spectrometry. Endurance sport samples demonstrated a significant increase of mainly medium-sized urinary proteins such as transferrin, zinc alpha-2-glycoprotein and prostaglandin H2 D-isomerase (30–80 kDa) in 2D-PAGE experiments. Proteinuria was evident in all samples after protein concentration measurements (protein/creatinine >15 mg/mmol). Alterations were also observed in strength sport samples, which showed an increase of low molecular weight proteins or protein fragments (<30 kDa, e.g., transthyretin, CD 59 antigen or an N-terminal transferrin fragment). In contrast, the concentration measurements did not imply proteinuria but total protein excretion was in a normal range.

The study provides a first overview on 2D maps of the urinary proteome after different types of exercise. Future studies may lead to the establishment of urinary protein maps that are typical for a certain type of sport or even an individual athlete. These maps may complement the blood passport of athletes in doping control.

Keywords: 2D-PAGE; doping; exercise; proteinuria; strength sports

Introduction

The presence of proteins in urine results from the balance of glomerular filtration and tubular reabsorption in the kidney. An approximate volume of 180 L of filtrate per day is produced by the glomeruli of both kidneys, which are perfused by approximately 1.3 L of blood every minute. Most of the filtrate is reabsorbed during the passage of the tubules and the resulting volume of urine is about 1.5 L per day.[1] The glomerular filtration barrier consists of three layers and the filtration of proteins through the wall is limited by size, charge and three-dimensional structure.[2] While low molecular-weight proteins can partially pass through the glomerular wall, intermediate-size proteins such as albumin or high molecular-weight proteins are mostly retained in circulation. Proteins that are filtered in the glomerulus are almost completely reabsorbed during the passage through the proximal tubulus. Tubular reabsorption is mainly accomplished by receptor-mediated endocytosis as long as the excretion is in a physiological range. It has been suggested that, if excessive amounts of protein are filtered, less specific mechanisms become involved.[3]

The physiological amount of daily excreted proteins is below 150 mg and the concentration of proteins in the urine varies widely because of the varying hydration status of the body. In addition to the proteins filtered from blood, proteins in urine may originate from the urinary tract. Proteinuria, defined as an excess of serum proteins in urine, is caused either by changes of the glomerular filter, the tubular reabsorption or a combination of both.[4] In clinical diagnostics proteinuria therefore can be characterized as tubular (predominantly small size proteins, <50 kDa), glomerular (medium molecular weight proteins, >50 kDa) or mixed type, depending on the molecular weight of the detected proteins.

The urinary proteome is influenced by exercise, and an increase in proteinuria was demonstrated to depend on degree and duration of exercise.[5,6] After a moderate workload, proteinuria consisted mainly of intermediately sized proteins while after strenuous exercise an additional component of small proteins was reported. This might be caused either by decreased reabsorption capacities of the tubule or overload of normal transport capacity of the proximal tubule by increased total protein load.[7] It was shown that work intensity has a higher impact on protein excretion than exercise duration, and the highest urinary protein

* *Correspondence to: Mario Thevis, Institute of Biochemistry/Center for preventive Doping Research, German Sport University Cologne, Am Sportpark Müngersdorf 6, 50933 Cologne, Germany. E-mail: thevis@dshs-koeln.de*

a *Institute of Biochemistry/Center for Preventive Doping Research, German Sport University Cologne, Germany*

b *Roche Pharma (Schweiz) AG, Reinach, Switzerland*

c *Laboratory Medicine, University Hospital, Basel, Switzerland*

concentrations were determined after 1–3 min of intense exercise while prolongation of the exercise reduced the protein content.[8,9]

Several pathophysiological mechanisms have been proposed to explain the phenomenon of post exercise proteinuria – for example, an increase of the filtration pressure of the glomeruli caused by increased cardiac output (1), conformation changes of serum proteins caused by lactic acidosis under exercise (2), increased venous pressure in the renal interstitium resulting in a decrease of tubular resorption (3), or overload of the tubular reabsorption due to increased filtration of the tubulus (4).

While mechanisms (1) and (2) result in a glomerular pattern, the third state results in a tubular pattern and the last mechanism in a mixed pattern of proteinuria. Since these elements may be altered differently depending on the type of sport, different patterns of proteinuria may be expected.

Several studies have explored urinary proteins; two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) maps were proposed[10] and proteins were identified by different purification, separation and mass spectrometry methods.[1,11–13]

In addition to clinical diagnosis, urine is the most common matrix for doping-control purposes and most of the test methods including detection of proteins such as erythropoietin, human chorionic gonadotropin or insulin are performed from urine samples.[14–17] Consequently, knowledge of urinary protein profiles of a specific subpopulation as represented by elite athletes is of utmost importance and may help to evaluate ambiguous or atypical results of sports drug-testing procedures for peptide hormones. Moreover, those 2D maps of the urinary proteome may complement the blood passport of athletes. Beside the doping topic, the profiles may provide information on the physiological state of an athlete in training and competition if future studies lead to individual or sports specific protein maps. In the present study, the urinary protein patterns of athletes belonging to different sport categories were compared to samples derived from a control group of healthy volunteers using 2D-PAGE. Proteins significantly differing within these populations were excised from 2D-gels and applied to trypsin digestion and subsequent mass spectrometric identification, to allow preliminary insights into possibly altered protein profiles characteristic of high-performance sportsmen.

Material and Methods

Amicon Ultra-15 (cutoff 10 kDa) and Ultrafree (cutoff 10 kDa) centrifugal filter devices were bought from Millipore (Billerica, MA, USA) and Bis-Tris SDS gels (12%), MOPS running buffer and lithium dodecyl sulfate (LDS) sample buffer were purchased from Invitrogen (Karlsruhe, Germany). IPG strips were bought from GE Healthcare (pH 4–7, Munich, Germany), Coomassie Blue stain was purchased from Pierce (Rockford, IL, USA) and Precision Plus Protein Standard was from Bio-Rad (Munich, Germany). Trypsin was obtained from Promega (Madison, WI, USA), DTT, acrylamide (both analytical grade) and all buffer ingredients (electrophoresis grade/analytical grade) as well as the Fluoro Profile Protein Quantification Kit were from Sigma (Deisendorf. Germany). Water, acetonitril and formic acid for nano-flow liquid chromatography were purchased from Biosolve (Valkenswaard, Netherlands).

Urine samples

Urine samples of three sport categories (endurance (Group E), strength (Group S) and team sport (Group T)) were obtained from in-competition doping controls after the samples were declared negative in all common sports drug testing procedures and athletes gave their written content that the samples may be used for research purposes. In-competition testing means that the samples are taken immediately after a race, which should be within one hour – this being the normal half life of exercise-induced proteinuria.[18] All samples were stored at +4 °C until analysis.

The control group urine specimens were collected from ten healthy volunteers who gave their written consent and occasionally exercised 1–4 times per week. The volunteers did not exercise directly prior to sampling. Endurance sport samples were obtained from cycling, biathlon and triathlon, strength sport samples from powerlifting and weightlifting, and team sports selected for the present study were ice hockey and soccer. All sample groups included five male and five female donors each. In all samples, total urinary protein (bezethoniumchloride method, Roche Ltd., Basel, Switzerland) and creatinine (enzymatic method, Wako Pure Chemical Industries Ltd., Osaka, Japan) were measured. Furthermore, protein amounts were approximated by measurement of the protein concentrations with a FuoroProfile Protein Quantification Kit from the concentrated urine samples to vary the amount of urine used for the 2D-PAGE.

To investigate the effect of different storage times of urine samples on the protein pattern, a blank urine sample was stored for 0, 1 and 4 weeks and the protein spot patterns after 2D-PAGE (in triplicate for each point of time) were compared by Image Master 2D Platinum software.

Sample preparation for 2D-PAGE

A volume of 15 mL of each urine sample was frozen at −80 °C and thawed to precipitate large solids. After centrifugation for 2 min at 4000 *g* the supernatant was transferred to an Amicon centrifugation filter (cutoff 10 kDa) and concentrated to a volume of approximately 200 µL. The retentate was transferred to an Ultrafree centrifugal filter and washed with MilliQ water until the conductivity was <300 µS. A volume of 200 µL of multichaotropic sample solution (MCSS, 7.7 M urea, 2.2 M thiourea, 4.4% CHAPS, 44 mM Tris) was added and the samples were centrifuged to a volume of 50 µL.

2D-PAGE

Approximately 300–600 µg of protein was prepared for the isoelectric focusing by addition of MCSS to a total volume of 100 µL, reduction of disulfide bonds with 15 µmol of DTT (45 min at room temperature (RT)) and derivatization of cysteine residues with acrylamide (45 µmol, 45 min at RT). After elimination of the acrylamide excess with a further 30 µmol of DTT (10 min at RT) and rehydration loading of the samples to the IPG strips (7 cm, pH 4–7), isoelectric focusing was performed overnight in an Ettan IPGphor 3 (GE Healthcare) with the following voltage gradient program: 100–300 V, 2 h; 300–10 000 V, 6 h; 10 000 V, until 80 000 Vh. The maximum current per strip was set to 50 µA. Strips were equilibrated two times for 10 min in LDS sample buffer and then applied to 8 cm, 4–12% Bis-Tris gels (XCell SureLock Mini-Cell (Invitrogen), SE 260 (GE Healthcare); 125 V, 90 min). After electrophoresis, gels were stained with Coomassie Blue and scanned on a light transmission scanner (GE Healthcare, 300 dpi, transparent mode, red filter).

2D-PAGE data analysis

Gels were evaluated using Image Master 2D Platinum software (GE Healthcare). Spots were aligned by definition of three landmarks and spots were matched prior to classification of the different groups. Each of the three sport categories was compared to the control group and spots with significant differences (Student t-test, $p = 0.05$) in their relative spot volume (spot volume in relation to the spot volume of all spots on the gel) were further analysed. Gels were also evaluated by visual inspection to take spots into consideration that were not recognized by the automated evaluation system. The software evaluation is based on the matching of spots in the same position of different gels. Accordingly, if the gels from one group or between the groups are too different, matching and therefore a software-based evaluation is impossible and an additional manual evaluation is required.

Trypsin digestion and mass spectrometry

Spots were excised and digested with trypsin overnight and peptides were extracted with 50 µL of 1% TFA, 50% acetonitrile. After evaporation and reconstitution, peptides were analysed by nano-UPLC-Orbitrap mass spectrometry. The Waters nano-UPLC instrument (Eschborn, Germany) was used with a Symmetry C18 precolumn (5 µm, 180 µm × 20 mm, flow 5 µm/min) and a BEH130C_{18} peptide column (1.7 µm. 100 µm × 100 mm) at a flow rate of 750 nL/min. A gradient program with 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B) was used as follows: 3 min 97% A, 3–5 min 80% A, 5–45 min 40% A, 45–48 min 20% A, 48–50 min 3% A, 50.01 min 97% A, 15 min equilibration. The LTQ Orbitrap (Thermo, Bremen, Germany) was used with a nanospray ion source in positive mode with 1.4 kV ionization voltage. Full scan spectra were recorded with a resolution of 60 000 (FWHM) and the collision energy for MS/MS experiments was set to 35% (arbitrary unit, Xcalibur 2.0 SR 2, Thermo) using helium as collision gas. The damping gas used was nitrogen supplied by a CMC nitrogen generator (CMC Instruments, Eschbach, Germany).

Identification of proteins in digested samples

For evaluation of the MS data, Bioworks 3.3 was used including the Swissprot/Uniprot database (Thermo, 2006). Variable search parameters were acrylamide alkylation of cysteine residues, allowance for one missed cleavage, and restriction to human proteins only. The identification of proteins was considered successful if at least three peptides were detected (peptide probability >30) and the molecular weight and isoelectric point was in accordance with the position of the selected spot on the gel except for modified (for example, if a protein is known to carry a glycoslyation) or fragmented proteins.

Results and Discussion

Urinary protein concentrations

Total urinary protein and creatinine concentrations were measured in all samples. All protein concentrations were normalized to creatinine and expressed as mg/mmol creatinine (Table 1). Mean protein concentrations in Group E and T were significantly higher than in the control group (72 ± 51.97 and 27 ± 16.35 mg/mmol creatinine versus 8 ± 5.21 mg/mmol creatinine; $p < 0.01$) whereas there was no significant difference between Group S (11 ± 9.2 mg/mmol creatinine) and controls ($p = 0.3$). The number of samples with urinary protein concentrations above 15 mg/mmol creatinine, which is considered as proteinuria was 0/10, 2/10, 5/10 and 10/10 in groups C, S, T and E, respectively.

Table 1. Protein concentrations and proteinuria in the different spots groups

Group	Samples with prot/crea >15 mg/mmol	Mean protein concentration mg/mmol creatinine (± SD)	P versus control
Control (C)	0/10	8 (± 5.21)	
Strength sport (S)	2/10	11 (± 9.22)	0.3
Team sport (T)	5/10	27 (± 16.35)	0.005
Endurance sport (E)	10/10	72 (± 51.97)	0.003

2D-PAGE for stability studies

The analysis of urine samples to investigate the effect of different storage times yielded no influence in samples prepared immediately or samples stored for 1 or 4 weeks at 4 °C. The analysis of 2D-gels did not show any spot that was significantly altered four weeks after storage.

2D-PAGE analysis of athlete's samples

Prior to 2D-PAGE, urinary protein amounts were measured with the FluoroProfile Protein Quantification Kit. Due to the measurement from the concentrates and the fact that the Kit is not tested for urine samples, the values are considered as approximations. This complicates the evaluation of the gels but the combination of software and visual analysis yields values that provide a good indication of differences between groups of gels in this pilot study. The spot volumes from the gels were normalized to the total amount of spots on the gel, which further yielded an acceptable normalization of the spot protein amount.

Using 2D-PAGE, several proteins were found to be different in sports and control urine specimens. In contrast, it was confirmed that no differences exist in the 2D-PAGE protein pattern of males and females of the control group as it was also described in an earlier study.[10]

In Figure 1, one representative gel from each group is depicted (all gels that were included in the evaluation are presented in the supplemental material). Due to very low protein content in some samples or poorly focused proteins, some gels were excluded from the evaluation. Finally, nine control samples, nine endurance sport samples, six strength sport samples and nine team sport samples were analysed. A considerable increase of albumin in comparison to other proteins was detected in most of the sports samples and was attributed to the change of the albumin charge and the alterations of the glomerular membrane after exercise.[2] Figure 1 illustrates the proteins that were significantly elevated in the in-competition sport samples after comparison to the control group (identified proteins, their sequence coverage, theoretical and experimental isoelectric point and molecular weight are given in the supplemental material). As individual protein spot volumes were normalized to the total protein on the gel, minor changes in the analyte spot volume may not be detected due to the relatively overrepresented increase of albumin excretion.

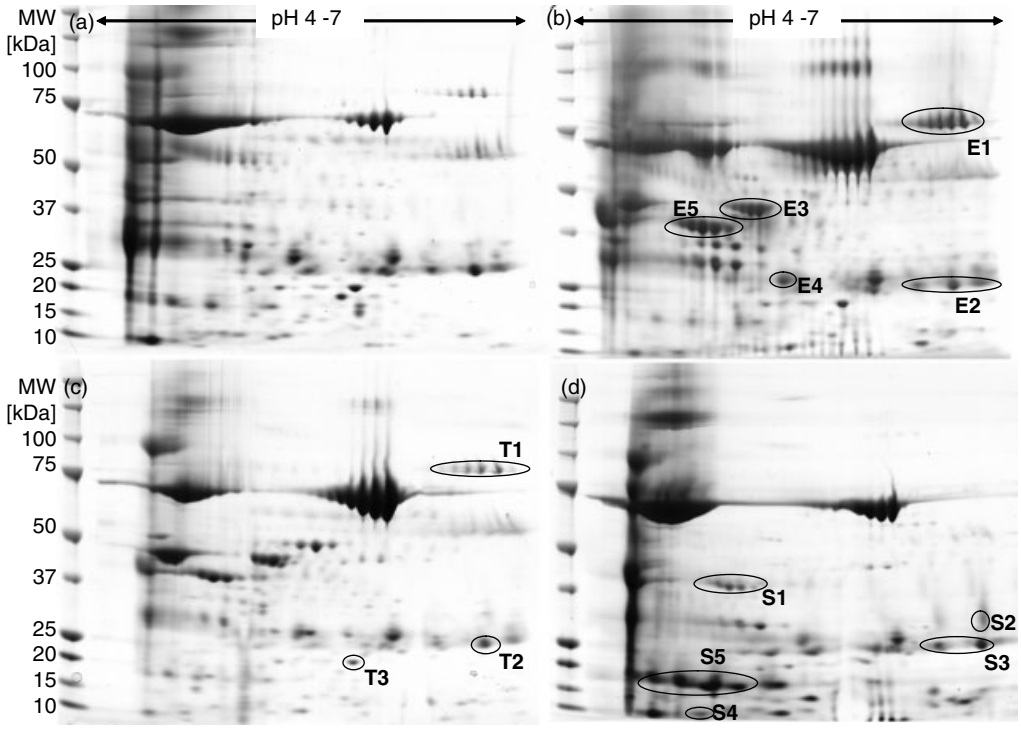

Figure 1. Exemplary 2D gels from the four analyzed groups: a) control group, b) endurance sport, c) team sport, d) strength sport. Encircled spots had an increased relative volume in comparison to the control group.

E1: Transferrin

E2: Immunoglobulin kappa chain (C-region), Prostaglandin H2 D-Isomerase

E3: Albumin (fragment), CD 14 antigen precursor

E4: Prostaglandin H2 D-isomerase, immunoglobulin kappa chain C-region

E5: Alpha-2-glycoprotein 1, gelsolin isoform b (fragment), CD 201 antigen, kininogen 1, clusterin isoform 1

T1: Transferrin, Polymeric immunoglobulin receptor

T2: Immunoglobulin kappa chain

T3: Heparan sulfate proteoglycan 2 (fragment)

S1: Alpha-2-glycoprotein 1, Gelsolin isoform b (fragment)

S2: Immunoglobulin gamma 1 heavy chain (fragment), Transferrin (fragment)

S3: Immunoglobulin kappa chain, Hemopexin (fragment), Albumin (fragment)

S4: Transthyretin

S5: CD 59 antigen, GM 2 ganglioside activator, Apolipoprotein A-I preproprotein

Therefore, significant differences in protein abundance that were observed in this study resulted from considerable changes of the qualitative renal protein excretion.

Distinct differences were detected in samples obtained from endurance sport athletes compared to those specimens originating from the control group. Likewise, team sport samples showed similarly deviating protein patterns in most of the gels. Spots E1 and T1 (Figure 1), which were significantly more abundant in endurance and team sport samples than in the control group, predominantly contained transferrin, and its appearance in normal physiological urine was reported earlier.[19] Spot E2 (Figure 1) was significantly elevated in endurance sport samples and contained immunoglobulin kappa chain (C-region) as well as prostaglandin H_2 D-isomerase. Its position on the gel is higher and more acidic than theoretical considerations would predict for prostaglandin H_2 D-isomerase, which is supposedly due to a glycosylation as described elsewhere.[20] Spots T2 and S3 (Figure 1) migrate to comparable positions on 2D-gels and contain the same immunoglobulin fragment. However, spot S3 was further composed of an N-terminal albumin fragment ending approximately at amino acid 264 and a C-terminal fragment of hemopexin. Another N-terminal fragment of albumin was detected in spot E3 (Figure 1) comprising the amino acid residues 1–403 and, thus, being much bigger than the fragment observed in spot S3. This spot (E3) additionally contains CD 14 antigen but in much lower amounts. Spot E4 was more intense in endurance sport samples and contains prostaglandin H_2 D-isomerase as well as immunoglobulin kappa chain. Spot E5, which was far more abundant in endurance samples than in controls and also elevated in strength sport samples (S1), contained predominantly zinc alpha-2-glycoprotein. One spot, labelled as T3 (Figure 1), was significantly elevated in team sport samples despite its generally low abundance and contained a C-terminal fragment of heparan sulfate proteoglycan 2. Strength sport samples yielded several additional spots and proteins, which were altered after exercise. Spot S2 contained a C-terminal fragment of immunoglobulin gamma 1 heavy chain starting approximately at amino acid 331 and a N-terminal fragment of transferrin (up to ~ aa 273). Spot S4 contained transthyretin and spot S5 was composed of CD 59 antigen as well as GM 2 ganglioside activator and apolipoprotein A-I. In general, strength sport samples showed relatively higher amounts of low molecular weight proteins, which are identified as

such, or fragments of medium and high molecular weight proteins (Figure 1). Excretion of low molecular weight proteins is usually connected to tubular dysfunction. This means that small proteins, which are usually readily reabsorbed by the tubulus, are excreted into the urine. It was proposed in the literature that post-exercise proteinuria might result mainly from elevated lactate levels.[5,9,21] However, in weightlifting and powerlifting competition, the exercise time is too short to represent a lactate-producing workload. Another possible factor that might influence proteinuria, is the increase of adrenergic activity. This is connected to higher blood pressure that is likely to be responsible for the elevated protein excretion, which is also considerably increased during weightlifting. Studies conducted on the blood pressure in bodybuilding found values as high as 345/245[22] or 370/360 mmHg.[23] If the increased blood pressure is the reason for a short tubular dysfunction, the time period of increased proteinuria is very short and the dilution effect resulting from a different time period since the last urination is much bigger than for the other groups. This might also explain the unchanged protein concentrations in comparison to the control group, which, at the same time, makes the qualitative differences after 2D-PAGE especially interesting. Intra-group comparison of the gels from strength sport samples (see supplemental material) outlined a wide heterogeneity within this group. The study of a larger population is therefore required to allow the identification of further differences.

Proteinuria in endurance sport has been analysed by several research groups in the past. Exercise intensity rather than the duration of workload was reported to increase proteinuria.[6,24] The present results substantiate this. Endurance sport samples in this study were obtained from athletes competing in triathlon and biathlon, indicating long competition distances and submaximal lactate levels. However, also team-sport samples yielded the expected result of higher total protein concentration and few increased protein abundances in the medium size range, which indicates an increased glomerular filtration that suits the nature of the exercise in this particular sporting category. The proteins identified are consistent with earlier urinary protein maps where most of the proteins were also detected,[1,10,11,13,19,25,26] although only one of these studies, conducted in 1968,[19] identified urinary proteins in post-exercise samples. Several fragments, especially from the strength sport samples, have not been reported before.

Conclusion

The results are an interesting starting point for further analysis of urinary protein patterns of different sport types as well as pre- and post-competition samples to monitor alterations in the protein profiles or even detect diagnostic proteins that are indicative for particular exercise regimens. The analysis of similar classes of samples on broad range IPG strips may provide even more information and discrimination power. The results should also be confirmed or determined more precisely when using the same protein amount per gel and strength sport samples may reach more significant differences if a bigger population of samples is analysed.

Acknowledgements

The study was carried out with financial support from the Manfred Donike Institute of Doping Analysis and the Federal Ministry of the Interior of the Federal Republic of Germany.

References

[1] J. Adachi, C. Kumar, Y. Zhang, J. V. Olsen, M. Mann, *Genome Biol.* **2006**, *7*, R80.
[2] B. Haraldsson, J. Nystrom, W. M. Deen, *Physiol. Rev.* **2008**, *88*, 451.
[3] M. Gekle, S. Mildenberger, R. Freudinger, S. Silbernagl, *Am. J. Physiol.* **1996**, *271*, F286.
[4] P. A. Peterson, P. E. Evrin, I. Berggard, *J. Clin. Invest.* **1969**, *48*, 1189.
[5] W. J. Montelpare, P. Klentrou, J. Thoden, *J. Sci. Med. Sport* **2002**, *5*, 219.
[6] J. R. Poortmans, *JAMA* **1985**, *253*, 236.
[7] J. R. Poortmans, L. Rampaer, J. C. Wolfs, *Eur J Appl Physiol Occup Physiol.* **1989**, *58*, 476.
[8] J. R. Poortmans, E. Blommaert, M. Baptista, M. E. De Broe, E. J. Nouwen, *Eur. J. Appl. Physiol. Occup. Physiol.* **1997**, *76*, 88.
[9] J. R. Poortmans, D. Labilloy, *Eur. J. Appl. Physiol. Occup. Physiol.* **1988**, *57*, 260.
[10] J. Oh, J. H. Pyo, E. H. Jo, S. I. Hwang, S. C. Kang, J. H. Jung, E. K. Park, S. Y. Kim, J. Y. Choi, J. Lim, *Proteomics* **2004**, *4*, 3485.
[11] V. Thongboonkerd, K. R. McLeish, J. M. Arthur, J. B. Klein, *Kidney Int.* **2002**, *62*, 1461.
[12] C. S. Spahr, M. T. Davis, M. D. McGinley, J. H. Robinson, E. J. Bures, J. Beierle, J. Mort, P. L. Courchesne, K. Chen, R. C. Wahl, W. Yu, R. Luethy, S. D. Patterson, *Proteomics* **2001**, *1*, 93.
[13] R. Pieper, C. L. Gatlin, A. M. McGrath, A. J. Makusky, M. Mondal, M. Seonarain, E. Field, C. R. Schatz, M. A. Estock, N. Ahmed, N. G. Anderson, S. Steiner, *Proteomics* **2004**, *4*, 1159.
[14] F. Lasne, L. Martin, N. Crepin, J. de Ceaurriz, *Anal. Biochem.* **2002**, *311*, 119.
[15] M. Kohler, C. Ayotte, P. Desharnais, U. Flenker, S. Lüdke, M. Thevis, E. Volker-Schänzer, W. Schänzer, *Int. J. Sports Med.* **2008**, *29*, 1.
[16] A. Thomas, M. Thevis, P. Delahaut, A. Bosseloir, W. Schänzer, *Anal. Chem.* **2007**, *79*, 2518.
[17] M. Thevis, A. Thomas, P. Delahaut, A. Bosseloir, W. Schänzer, *Anal. Chem.* **2006**, *78*, 1897.
[18] J. R. Poortmans, *Sports Med.* **1984**, *1*, 125.
[19] J. Poortmans, R. W. Jeanloz, *J. Clin. Invest.* **1968**, *47*, 386.
[20] P. Lescuyer, A. Gandini, P. R. Burkhard, D. F. Hochstrasser, J. C. Sanchez, *Electrophoresis* **2005**, *26*, 4563.
[21] J. R. Poortmans, M. Jourdain, C. Heyters, F. D. Reardon, *Can. J. Sport. Sci.* **1990**, *15*, 126.
[22] P. Palatini, L. Mos, L. Munari, F. Valle, M. Del Torre, A. Rossi, L. Varotto, F. Macor, S. Martina, A. C. Pessina, C. D. Palu, *J. Hypertens Suppl.* **1989**, *7*, S72.
[23] J. A. Narloch, M. E. Brandstater, *Arch. Phys. Med. Rehabil.* **1995**, *76*, 457.
[24] J. R. Poortmans, N. Mathieu, P. De Plaen, *Eur. J. Appl. Physiol. Occup. Physiol.* **1996**, *72*, 522.
[25] P. G. Zerefos, K. Vougas, P. Dimitraki, S. Kossida, A. Petrolekas, K. Stravodimos, A. Giannopoulos, M. Fountoulakis, A. Vlahou, *Proteomics.* **2006**, *6*, 4346.
[26] M. Soldi, C. Sarto, C. Valsecchi, F. Magni, V. Proserpio, D. Ticozzi, P. Mocarelli, *Proteomics* **2005**, *5*, 2641.