Review

Applicability of 2D gel electrophoresis and liquid chromatography in proteomic analysis of urine using mass spectrometry MALDI-TOF

T. Banach[1], Ł. Adaszek[1], D. Wyłupek[2], M. Winiarczyk[3], S. Winiarczyk[1]

[1] Department of Epizootiology and Clinic of Infectious Diseases, University of Life Sciences, Głęboka 30, 20-612 Lublin, Poland,
[2] Department and Clinic of Animal Internal Diseases, University of Life Sciences, Gleboka 30, 20-612 Lublin, Poland
[3] Department of Vitreoretinal Surgery, Medical University of Lublin, Chmielna 1, 20-079 Lublin, Poland

Abstract

Proteomics including the studies of the structure, function and dependences between proteins is more and more extensively applied in human medicine and veterinary medicine. The analysis of protein profiles of tissues and body fluid from healthy and ill individuals allows to identify diagnostic, prognostic and predictive markers in various pathological states in people and animals. This paper presents preparation of urine samples for analysis in the mass spectrometer MALDI-TOF (Ultraflextreme, Bruker, Bremen, Germany) by means of two methods: liquid chromatography based on the system Nano-LC (PROTEINER FC II, Bruker Daltonics, Bremen Germany). and two-direction electrophoresis 2DE (GE Healthcare, United Kingdom). Both methods enable separation of the mixture under consideration into individual fractions of high purity indispensable for obtaining readable mass spectra. The purpose of this paper is to determine applicability of these methods in analysis of protein composition of urine samples.

Key words: chromatography, protemic, mass spectrometry, urine

Introduction

Proteomics including the studies of structure, function and dependences between proteins is more and more extensively applied in human medicine and veterinary medicine. The analysis of protein profiles of tissues and body fluid from healthy and ill individuals allows to identify diagnostic, prognostic and predictive markers in various pathological states in people and animals.

In Veterinary Medicine proteomic studies were used for diagnosis of numerous, especially subclinical, diseases. For example, in the last few years comparative proteomics was used to evaluate milk proteome

---

Table 1. Proteins pathognomic for some diseases.

Disease	Identified
Liddle's disease	Beta and gamma epithelia sodium ducts
Kidney proximal duct hyperoxemia	Carbonate anhydrase IV
Autosomal dominant and autosomal recessive nefrogene diabetes	Aquaporine
Gitelman symptom	Thiazide-sensitive NaCl cotransporter
Familial kidney hypomagnesemia	Transport regulator

profiles during acute and chronic phases of bacterial intramammary infection. The results of these researches provide the important information for therapy of these conditions (Addis et al. 2011, Kim et al. 2011, Barreiro et al. 2012, Chiaradia et al. 2013).

Urine is an excrement containing useless or harmful metabolic products which can be a source of useful biomarkers (Marimuthu et al. 2011). Its proteins can be analyzed directly in the native sample and also after prior separation of solid fraction by means of centrifugation. Studies of urine proteome give important insight into health status of the body. The obtained results can be used in diagnostics and treatment of various diseases, such as bladder cancer, ovarian cancer, progressive glomerular disease, renal cell cancer or gastric cancer (D'Amico et al. 2003, Khan et al. 2005, Holt et al 2006, Shigaki et al. 2006, Salo et al. 2007, Yang et al. 2009, Saito et al. 2010, Welton et al. 2010, Jansen et al. 2011, Liu et al. 2012, Nabity and Lees 2012, Wu et al. 2012). High sensitivity and resolution of available analytical methods allows listing the proteins present in the urine including those at low concentrations and making a map for each pathological state (Anderson et al. 1979). At present it is used in many fields of medicine e.g. in nephrology, where the analysis allows for early diagnosis of nephropathy before clinical symptoms occur. Single, pathognomonic proteins also occur in urine in specific pathological states (Table 1) (Vlahou et al. 2001, Pang et al. 2002). Searching for protein markers in urine in some diseases is challenging for modern clinical diagnostics (Khan and Packer 2006). The investigation require preparation of biological material sample, its analysis using advanced research techniques (chromatography, 2D electrophoresis) and then associating the obtained results with symptoms (Khan and Packer 2006). The reliability of the obtained results depends on many factors i.e. the way of material uptake, its storage, analysis method, reagents purity, quality of analytical apparatus etc.

Urine collection for proteomic studies

The reliable result of proteomic studies is the collection of urine sample qualitatively consistent with the composition of eventual urine in the urinary bladder. The simplest and non-invasive method is urine collection from the mid-stream micturition into the sterile containers (Nabity et al. 2011). The 5-20 ml sample is centrifuged at 3000 g for 5 minutes. Then, the inhibitors mixture is added to the obtained supernatant to prevent the proteins decomposition. Their concentration is determined by the Bradford method and the whole content is stored at -80ºC for further analyses (Zhou et al. 2006). To avoid the risk of sample contamination with outer environmental factor, in some cases cystocentis, the direct puncture of the urine bladder, is carried out with monitoring by USG. The disadvantage of this method is invasive propensity and necessity of performing it in the clinic by the doctor. Catheterization of the patient is a less invasive method. However, urine obtained in this way can be contaminated in the process of a catheter being introduced to the bladder with e.g. bacteria from outer sexual organs or laminated epithelia (Bubenik et al. 2007). In every case, day and night fluctuations affect the interpretation of results.

2D Gel Electrophoresis

The first step in preparation of the urine sample for proteins separation by means of 2D electrophoresis is proper preparation of the material including: filtration to purify the sample from interferents of the mass <3kDa, concentration of the sample e.g. by precipitation with acetone and then (usually during the night) centrifugation (Khan and Packer 2006, Konvalinka et al. 2012). TBT is added to the sample to reduce proteins and medium alkalization takes place after the addition of acrylamide. To remove salt, the material must be filtered in the desalination column

but with small volume of the sample, it is difficult to choose proper separation time on the column. To make this stage of investigations more efficient, the column should be coupled with the spectrometer or a small-mass marker, which will appear as the first in the effluent should be used. It should eluate close to the retention time of the first of the proteins to wash out all interferents of the mass close to the lightest protein with its simultaneous "rescue" (Vaezzadeh et al. 2010, Court et al. 2011). An important factor affecting the quality of the sample prepared for 2D electrophoresis is its conductivity. It decides about the separation effectiveness. The recommended conductivity is 300 µS/cm (Khan and Packer 2006), whereby the urine samples e.g. in people had different conductivity depending on collection time. Even after acetone washing aimed at the sample concentration conductivity of urine, e.g. in people, decreased insignificantly (Khan et al. 2005).

In order to standardize the analysis method, urine should be taken up from mid-stream in the morning (Khan et al. 2005, Khan and Packer 2006, Thongboonkerd 2007, Benkali et al. 2008). This allows to avoid taking up a large amount of already mentioned interferents appearing in the first fraction of excreted urine (Theodorescu and Mischak 2007, Thongboonkerd and Saetun 2007). However, this does not help get rid of the problem. In fact, "pure" urine without cellular elements can be obtained by urine bladder catheterization. The disadvantage of this sample collection method, besides invasive propensity, is the fact that the stress can cause changes in the urine composition (Bubenik et al. 2007).

The advantage of 2D electrophoresis as a method of preparation of proteins in the urine sample for analysis by means of mass spectrometry MALDI-TOF is accuracy of protein separation not only with respect to the isoelectric point (first direction), but also as the function of mass (second direction) (Weeks 2010). A sample of urine is not a complicated mixture in which most compounds have similar mass. If that was a case, after the whole process a mixture of several components of similar composition would be obtained on one spectral line. Some standards are available commercially which allegedly make quantitative determination possible. If the range of isoelectric points is known, zooming is possible thus making separation of compounds mixture accurate (Mischak et al. 2007, Thongboonkerd 2007, Thongboonkerd and Saetun 2007). Its disadvantage is a lack of possibility to use such concentration during the standard measurement in the range of pH 3-10. The disadvantage of electrophoresis as a method of preparation of urine sample for proteomic analysis is necessity of preparation of starting material characterized by minimal

threshold concentration depending on the quantity of strips and gels applied by the user. The example of such concentration can be results presented by Khan and Packer (2006), where the authors assume the necessity of a minimum concentration of 200 µg/20 ml of the initial urine for analysis. In the case of the samples taken from healthy animals, this criterion cannot always be met due to individual characteristics e.g. dog weight, specific metabolism. Another limitation of electrophoresis as a method of sample preparation is necessity of elimination of compounds which appear in diseases e.g. oxalates or calcium phosphates in nephrolithiasis. However, the most significant disadvantage of gel 2D electrophoresis are losses of biological material which is not retained in the gel or is washed out from it during the whole process of separation (Pieper et al. 2004, Weeks 2010). This is of significant importance in the analysis of compounds, which, when occurring in small amounts in the sample, can get lost. An important factor affecting the quality of mixture components separation by means of gel 2D electrophoresis is application of standardized procedures which assures the reproducibility of results (Zerefos and Vlahou 2008). A lack of convergence between the results obtained within a few days of conducting the same process in the reproducible conditions is quite frequent (Sobhani 2010). Due to the character of biological samples, it is essential that the gel temperature does not exceed that of protein denaturation which may occur with the local increase of substance density composition which results in increase of electric current resistance and which is directly connected with temperature conditions affecting the sample (Sobhani 2010, Weeks 2010).

Liquid Chromatography

Liquid chromatography is an alternative for the two-direction gel electrophoresis (Liu et al. 2012). Using this method it is possible to eliminate some disadvantages of 2D electrophoresis what can be shown on examples based on Nano-LC chromatography. Chromatography used as a method of separation of urine protein mixtures for qualitative determination by means of mass spectrometry MALDI-TOF (Lapolla et al. 2006, Sun et al. 2006, Bryan et al. 2011). In these papers the required amount of proteins for determination is 2 µg/5 ml of collected urine. According to the requirements of the producer the samples to be analyzed by the MALDI-TOF mass spectrometry must be filtered to separate ionic compounds, heavy metals, compounds of mass < 1 kDa from the protein (Saito et al. 2010, Cobzac and Gocan

2011). "Light" fraction can be qualitatively analyzed thanks to a special procedure (Norden et al. 2004) and modification of detection method which is impossible in the case of 2D electrophoresis. Owing to the use of a precolumn of the SCX type in the Nano-LC system it is possible to separate over 2.5 thousand compounds from mixture. (Machtejevras et al. 2009, Balog et al. 2010). This procedure linked with the use of analytical column with the C-18 phase of the 25 cm length enables better separation than the comparable gel 2D electrophoresis system. However, liquid chromatography used as a method of preparation of a sample for analysis has also its own limitations (Erni 1982, Adachi et al. 2006, De Jong et al. 2010). One of the main assumptions in sample preparation for analysis is adjustment of the number of theoretical shelves to applied mobile phase. The aim of such procedure is determination of time needed for spreading the sample on the plate spot so that there is a small amount of mixture components as possible. The ideal situation would be such ratio of the dosing time to the retention time of the studied substance that there was only this one on a given spot. When this is not possible the mobile and dropping time should be chosen so that there is possibility the smallest number of mixture components on a given spot washed out from column and their spectrum was on one spot but not repeated in the dosage series (Erni 1982). Applying a MALDI-TOF mass spectrometer as an analyzer and procedures connected with its operations, it is possible to eliminate one of the disadvantages of liquid chromatography which is evolution of separated compounds from the mobile phase. The system of linked Nano-LC and MALDI-TOF mass spectrometer enables, besides separation of mixture components, preparation of an almost perfectly pure sample after evaporation of solvent being components of mobile phase which can be subjected for further analysis (Suzuki et al. 2007). This is an obvious advantage over the gel electrophoresis methods where after the separation of mixture components it is necessary to use the procedure to transfer the sample from gel the solvent, then to evaporate the solvent by means of mass spectrometry MALDI-TOF. However, one should bear in mind that the disadvantage of liquid chromatography compared to the electrophoretic methods is the effect of sample viscosity on the pressure which must be applied so that a multi-component mixture could be separated. If compounds of similar mass make a large part of sample they will eluate from the column in similar period of time and the effect of this there will be heterogeneity of composition and viscosity of a sample. This may decrease quality of mixture separation and a sample purity.

Conclusions

The above presentation of advantages and disadvantages of gel 2D electrophoresis and liquid chromatography as the methods used for purification and separation of urine proteins show that there is no perfect method for preparation and separation of components of biological material. Both techniques are characterized by disadvantages which limit preparation of perfectly pure material for analysis. To evade them it is recommended to link them separating the prepared mixture in 1D electrophoresis and then extracting proteins from the cut strip fragments for separation by means of liquid chromatography. The purified material obtained in this way is a sample ready for analysis by means of mass spectrometry.

References

Adachi J, Kumar C, Zhang Y, Olsen JV, Mann M (**2006**) The human urinary proteome contains more than 1500 proteins, including a large proportion of membrane proteins. Genome Biol 7: R80.

Addis MF, Pisanu S, Ghisaura S, Pagnozzi D, Marogna G, Tanca A, Biosa G, Cacciotto C, Alberti A, Pittau M, Roggio T, Uzzau S (**2011**) Proteomics and pathway analyses of the milk fat globule in sheep naturally infected by Mycoplasma agalactiae provide indications of the in vivo response of the mammary epithelium to bacterial infection. Infect Immun 79: 3833-3845.

Anderson NG, Anderson NL, Tollaksen SL, Hahn H, Giere F, Edwards J (**1979**) Analytical techniques for cell fractions: XXV. Concentration and two-dimensional electrophoretic analysis of human urinary proteins. Anal Biochem 95: 48-61.

Balog CI, Hensbergen PJ, Derks R, Verweij JJ, Van Dam GJ, Vennervald BJ, Deelder AM, Mayboroda OA (**2009**) Novel automated biomarker discovery work flow for urinary peptidomics. Clin Chem 55: 117-125.

Barreiro JR, Braga PA, Ferreira CR, Kostrzewa M, Maier T, Wegemann B, Böettcher V, Eberlin MN, dos Santos MV (**2012**) Nonculture-based identification of bacteria in milk by protein fingerprinting. Proteomics 12: 2739-2745.

Benkali K, Marquet P, Rérolle J, Le Meur Y, Gastinel L (**2008**) A new strategy for faster urinary biomarkers identification by Nano-LC-MALDI-TOF/TOF mass spectrometry. BMC Genomics 9: 541.

Bryan RT, Wei W, Shimwell NJ, Collins SI, Hussain SA, Billingham LJ, Murray PG, Deshmukh N, James ND, Wallace DM, Johnson PJ, Zeegers MP, Cheng KK, Martin A, Ward DG. (**2011**) Assessment of high-throughput high-resolution MALDI-TOF-MS of urinary peptides for the detection of muscle-invasive bladder cancer. Proteomics Clin Appl 5: 493-503.

Bubenik LJ, Hosgood GL, Waldron DR, Snow LA (**2007**) Frequency of urinary tract infection in catheterized dogs and comparison of bacterial culture and susceptibility testing results for catheterized and noncatheterized dogs with urinary tract infections. J Am Vet Med Assoc 231: 893-899.

Chiaradia E, Valiani A, Tartaglia M, Scoppetta F, Renzone G, Arena S, Avellini L, Benda S, Gaiti A, Scaloni A (**2013**) Ovine subclinical mastitis: Proteomic analysis of whey and milk fat globules unveils putative diagnostic biomarkers in milk. J Proteomics 83: 144-159.

Cobzac SC, Gocan S (**2011**) Sample preparation for high performance liquid chromatography: Recent progress. J Liq Chromatogr Relat Technol 34: 1157-1267

Court M, Selevsek N, Matondo M, Allory Y, Garin J, Masselon CD, Domon B (**2011**) Toward a standardized urine proteome analysis methodology. Proteomics 11: 1160-1171.

D'Amico G, Bazzi C (**2003**) Pathophysiology of proteinuria. Kidney Int 63: 809-825.

De Jong WH, Wilkens MH, De Vries EG, Kema IP (**2010**) Automated mass spectrometric analysis of urinary and plasma serotonin. Anal Bioanal Chem 396: 2609-2616.

Erni F (**1982**) Liquid chromatography-mass spectrometry in the pharmaceutical industry: Objectives and needs. J Chromatogr 251: 141-151.

Holt GE, Schwartz HS, Caldwell RL (**2006**) Proteomic profiling in musculoskeletal oncology by MALDI mass spectrometry. Clin Orthop Relat Res 450: 105-110.

Jansen RS, Rosing H, Schellens JH, Beijnen JH (**2011**) Mass spectrometry in the quantitative analysis of therapeutic intracellular nucleotide analogs. Mass Spectrom Rev 30: 321-343.

Jeong SH, Hyo JP, Jae HJ, Sung CK, Hyung CP, Choong WK, Kee RK, Ky HC (**2005**) Identification of proteins differentially expressed in the conventional renal cell carcinoma by proteomic analysis. J Korean Med Sci 20: 450-455.

Khan A, Grinyer J, Truong ST, Breen EJ, Packer NH (**2005**) New urinary EPO drug testing method using two-dimensional gel electrophoresis. Clin Chim Acta 358: 119-130.

Khan A, Packer NH (**2006**) Simple urinary sample preparation for proteomic analysis. J Proteome Res 5: 2824-2838.

Kim Y, Atalla H, Mallard B, Robert C, Karrow N (**2011**) Changes in Holstein cow milk and serum proteins during intramammary infection with three different strains of Staphylococcus aureus. BMC Vet Res 7: 51

Konvalinka A, Scholey JW, Diamandis EP (**2012**) Searching for new biomarkers of renal diseases through proteomics. Clin Chem 58: 353-365.

Lapolla A, Fedele D, Seraglia R, Traldi P (**2006**) The role of mass spectrometry in the study of non-enzymatic protein glycation in diabetes: an update. Mass Spectrom Rev 25: 775-797.

Liu W, Liu B, Cai Q, Li J, Chen X, Zhu Z (**2012**) Proteomic identification of serum biomarkers for gastric cancer using multi-dimensional liquid chromatography and 2D differential gel electrophoresis. Clin Chim Acta 413: 1098-1106.

Machtejevas E, Marko-Varga G, Lindberg C, Lubda D, Hendriks R, Unger KK (**2009**) Profiling of endogenous peptides by multidimensional liquid chromatography: On-line automated sample cleanup for biomarker discovery in human urine. J Sep Sci 32: 2223-2232.

Marimuthu A, O'Meally RN, Chaerkady R, Subbannayya Y, Nanjappa V, Kumar P, Kelkar DS, Pinto SM, Sharma R, Renuse S, Goel R, Christopher R, Delanghe B, Cole RN, Harsha HC, Pandey A (**2011**) A Comprehensive Map of the Human Urinary Proteome. J Proteome Res 10: 2734-2743.

Mischak H, Julian BA, Novak J (**2007**) High-resolution proteome/peptidome analysis of peptides and low-molecular-weight proteins in urine. Proteomics Clin Appl 1: 792.

Nabity MB, Lees GE, Dangott LJ, Cianciolo R, Suchodolski JS, Steiner JM (**2011**) Proteomic analysis of urine from male dogs during early stages of tubulointerstitial injury in a canine model of progressive glomerular disease. Vet Clin Pathol 40: 222-236.

Nabity MB, Lees GE, Cianciolo R, Boggess MM, Steiner JM, Suchodolski JS (**2012**) Urinary biomarkers of renal disease in dogs with X-linked hereditary nephropathy. J Vet Intern Med 26: 282-293.

Norden AG, Sharratt P, Cutillas PR, Cramer R, Gardner SC, Unwin RJ (**2004**) Quantitative amino acid and proteomic analysis: very low excretion of polypeptides >750 Da in normal urine. Kidney Int 66: 1994-2003.

Pang JX, Ginanni N, Dongre AR, Hefta SA, Opitek GJ (**2002**) Biomarker discovery in urine by Proteomics. J Proteomic Res 1: 161-169.

Pieper R, Gatlin CL, McGrath AM, Makusky AJ, Mondal M, Seonarain M, Field E, Schatz CR, Estock MA, Ahmed N, Anderson NG, Steiner S (**2004**) Characterization of the human urinary proteome: a method for high-resolution display of urinary proteins on two-dimensional electrophoresis gels with a yield of nearly 1400 distinct protein spots. Proteomics 4: 1159-1174.

Saito K, Yagi K, Ishizaki A, Kataoka H (**2010**) Determination of anabolic steroids in human urine by automated in-tube solid-phase microextraction coupled with liquid chromatography-mass spectrometry. J Pharm Biomed Anal 52: 727-733.

Salo PK, Vilmunen S, Salomies H, Ketola RA, Kostiainen R (**2007**) Two-dimensional ultra-thin-layer chromatography and atmospheric pressure matrix-assisted laser desorption/ionization mass spectrometry in bioanalysis. Anal Chem 79: 2101-2108.

Shigaki S, Sonoda T, Nagashima T, Okitsu O, Kita Y, Niidome T, Katayama Y (**2006**) A new method for evaluation of intracellular protein signals using mass spectrometry. Sci Tech Adv Mater 7: 699-704.

Sobhani K (**2010**) Urine proteomic analysis: use of two-dimensional gel electrophoresis, isotope coded affinity tags, and capillary electrophoresis. Methods Mol Biol 641: 325-346.

Sun W, Gao S, Wang L, Chen Y, Wu S, Wang X, Zheng D, Gao Y (**2006**) Microwave-assisted protein preparation and enzymatic digestion in proteomics. Mol Cell Proteomics 5: 769-776.

Suzuki Y, Miyazaki M, Ito E, Suzuki M, Yamashita T, Taira H, Suzuki A (**2007**) Structural characterization of N-glycans of cauxin by MALDI-TOF mass spectrometry and nano LC-ESI-mass spectrometry. Biosci Biotechnol Biochem 71: 811-816.

Theodorescu D, Mischak H (**2007**) Mass spectrometry based proteomics in urine biomarker discovery. World J Urol 25: 435-443.

Thongboonkerd V, Saetun P (**2007**) Bacterial overgrowth affects urinary proteome analysis: recommendation for centrifugation, temperature, duration, and the use of preservatives during sample collection. J Proteome Res 6: 4173-4181.

Thongboonkerd V (**2007**) Practical points in urinary proteomics. J Proteome Res 6: 3881-3890.

Vaezzadeh AR, Briscoe AC, Steen H, Lee RS (**2010**) One-step sample concentration, purification, and albumin depletion method for urinary proteomics. J Proteome Res 9: 6082-6089.

Vlahou A, Schellhammer PF, Mendrinos S, Patel K, Kondylis FI, Gong L, Nasim S, Wrigh Jr GL Jr (**2001**) Development of a novel proteomic approach for the detection of transitional cell carcinoma of the bladder in urine. Am J Pathol 158: 1491-1502.

Weeks ME (**2010**) Urinary proteome profiling using 2D-DIGE and LC-MS/MS. Methods Mol Biol 658: 293-309.

Welton JL, Khanna S, Giles PJ, Brennan P, Brewis IA, Staffurth J, Mason MD, Clayton A (**2010**) Proteomics analysis of bladder cancer exosomes. Mol Cell Proteomics 9: 1324-1338.

Wu S, Xu K, Chen G, Zhang J, Liu Z, Xie X (**2012**) Identification of serum biomarkers for ovarian cancer using MALDI-TOF-MS combined with magnetic beads. Int J Clin Oncol 17: 89-95.

Yang Y, Zhao S, Fan Y, Zhao F, Liu Q, Hu W, Liu D, Fan K, Wang J, Wang J (**2009**) Detection and identification of potential biomarkers of non-small cell lung cancer. Technol Cancer Res Treat 8: 455-466.

Zerefos PG, Vlahou A (**2008**) Urine sample preparation and protein profiling by two-dimensional electrophoresis and matrix-assisted laser desorption ionization time of flight mass spectroscopy. Methods Mol Biol 428: 141-157.

Zhou H, Pisitkun T, Aponte A, Yuen PS, Hoffert JD, Yasuda H, Hu X, Chawla L, Shen RF, Knepper MA, Star RA (**2006**) Exosomal Fetuin-A identified by proteomics: a novel urinary biomarker for detecting acute kidney injury. Kidney Int 70: 1847-1857.