Chapter 4
Human Urine Proteome: A Powerful Source for Clinical Research

Lili Zou and Wei Sun

Abstract As noninvasive and easily available biological fluid, urine is becoming an ideal sample for proteomic study. In recent years, researchers endeavored in profiling urinary proteome and discovering potential disease biomarkers. However, there are still many challenges in the studies of urinary proteome for the complexity of urine. In this article, we review current status of urinary sample preparation, including collection, storage, and extraction of urinary proteins, and the overall urinary proteome analysis so far, which may be helpful for urinary proteome analysis.

Keywords Urinary proteome · Mass spectrometry · Preparation and storage · Normal human

4.1 Introduction

Body fluids have been regarded as the significant source of biomarkers, which could be used for the early diagnosis and state forecast of clinical diseases [1–4]. As an easily obtainable biological fluid, urine plays a crucial role in the clinical proteomics.

Urine is excreted by the kidney to eliminate waste products from plasma. About 150–180 L of plasma is filtered by glomeruli to develop the "primitive urine." More than 99 % of "primitive urine" is reabsorbed by the renal tubule, and the "final urine" is remained to be excreted [5]. Approximately 30 % of urinary proteins originate from the plasma proteins, whereas 70 % comes from the kidney and the urinary tract [6, 7]. Therefore, the urinary proteome might supply important biomarkers directly reflecting the functions of the kidney and related organs [8].

L. Zou · W. Sun (✉)
Core Facility of Instrument, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College, Dong Dan San Tiao, Beijing 100005, China
e-mail: sunwei1018@sina.com

Y. Gao (ed.), *Urine Proteomics in Kidney Disease Biomarker Discovery*, Advances in Experimental Medicine and Biology 845,
DOI 10.1007/978-94-017-9523-4_4

Urine has several advantages to discover biomarkers of diseases: (1) It is easy to be collected in large amount and noninvasive way and (2) urinary samples are less complex than plasma and carry many proteins, peptides, and amino acids that have not been discovered in plasma [9]. Therefore, many researchers did their best to have a deeper understanding of urinary proteins and discover potential biomarkers in recent years.

However, there still are many difficulties and problems needed to further explore and study. In this review, we summarize achievements of urinary proteomics, including sample collection, preparation, and urinary proteome analysis in recent years, which may be helpful for further studies.

4.2 Collection and Storage

4.2.1 The Types of Urine

In clinic, many kinds of urinary samples have been used according to the different examination. However, many factors may influence the components of urinary proteome [10], including daily activities, physiological variations, and environmental factors such as temperature and pH. Therefore, several types of urinary samples have been analyzed by proteomic approach, including first-morning urine, 24-h urine, second-morning urine, random urine, and the urine collected after drinking a large amount of water or after drinking coffee.

Twenty-four-hour urine can show the excretion of urinary proteins within a day [11]. But the collection of 24-h urine depends on patient compliance, which is unpractical to be completed entirely and easy to have some errors during the collection process [12]. Concerning first-morning urine, it cannot exhibit urine "diurnal variation" (different time-points' variation in all day) [11]. And Hoorn et al. [13] reported that first-morning urine may have bacterial contamination due to the long residence time of bladder.

Sun et al. [10] made a qualitative and quantitative analysis of five samples (first-morning void, second-morning void, excessive water-drinking void, random void, and 24-h void) collected in 1 day from healthy volunteers by 1-D LC/MS. They found no significant differences in the protein numbers of these five samples, and 42 common proteins to five samples contributed an average of 88.7 % of abundance to each sample. Thongboonkerd et al. [14] compared four different time-points urine, including first-morning urine, afternoon urine, water-loading urine, and urine after drinking a cup of coffee. They found the first-morning urine contained greater amount of proteins, but less protein spots visualized in 2D gel than afternoon urine. The water-loading urine had the least amount of proteins by 2D-PAGE analysis, but exhibited a few newly presenting spots. There were more spots in the sample after caffeine ingestion than in water-loading urine.

To avoid bacterial contamination deriving from skin contamination, midstream urine is usually recommended as the standard for urinary proteome analysis, particularly for women [15]. Schaub et al. [16] employed surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) in profiling the first-stream urine and midstream urine from three females and three males. For male samples, there were no observable differences between midstream and first-void urines. But for female samples, first-void urine emerged three specific SELDI peptide peaks after 3-day storage compared with midstream urine.

4.2.2 Protease Inhibitor

Protease inhibitors had been initially suggested to be used to prevent proteolysis of clinical biological fluids, which was caused by endogenous proteases [17]. However, whether protease inhibitors are necessary for the studies of urinary proteomics is debatable. Shinada et al. [18] incubated 30-kDa nonglycosylated [^{125}I]IGFBP-3 with urinary samples and found IGFBP-3 proteolysis by SDS-PAGE analysis. But Havanapan et al. [17] studied the effect of protease inhibitors cocktail on midstream random urinary specimens and found that no observable qualitative and quantitative changes by two-dimensional gel electrophoresis (2-DE) analysis. Thongboonkerd [11] suggested protease inhibitors were unnecessary for the studies of nonproteinuric urine because there were lower amount of proteases in urine than in plasma, cells, or tissues.

4.2.3 Preservatives

During the storage, urinary samples might have bacterial overgrowth, which could change the urinary proteome [19]. Therefore, preservatives were recommended to prevent the bacterial overgrowth after collection [19]. Thongboonkerd and Saetun [19] studied the effects of the addition of either sodium azide (NaN_3) or boric acid on bacterial overgrowth in pooled urine from 5 healthy individuals. They found the addition of NaN_3 and boric acid could delay the bacterial overgrowth, and greater delay (for at least 48 h) was obtained by relatively higher preservatives. They recommended addition of 2–20 mM boric acid or 0.1–1 mM NaN_3 to one-void random urinary specimens and addition of 200 mM boric acid or 10 mM NaN_3 to 24-h urinary collections.

4.2.4 Storage Temperature

Appropriate storage temperature could decrease degradation of urinary proteins to some extent. In 1999, Klasen et al. [20] analyzed changes of albumin concentrations after urinary samples were stored at 4, −20, and −70 °C. They found that if samples could be analyzed in 4 weeks after collected, the best storage temperature was 4 °C. And they recommended −70 °C was used as storage temperature for longer storage. They did not suggest storage temperature −20 °C because they found the IgG concentrations decreased after 1-week storage at −20 °C. In 2007, Thongboonkerd and Saetun [19] reported that to prevent the bacterial overgrowth, uncentrifuged urinary samples without preservative should be kept no longer than 20 h at 4 °C. Without any preservative, urinary samples should not be stored at room temperature for longer than 8 h.

4.2.5 Freeze–Thaw Cycle

Previous studies showed freeze–thaw cycles could influence the components of body fluid proteome, including serum/plasma and cerebrospinal fluid [21, 22]. Studies also showed that when urinary samples were stored at low temperature, researchers should avoid freeze–thaw cycles [11]. Schaub et al. [16] analyzed first-void and midstream urinary specimens from three females and three males by SELDI-TOF MS. Results indicated that after 1–4 freeze–thaw cycles, the urinary proteome did not change remarkably except for the loss of intensity in some peaks, whereas some small peaks were undetectable after the fifth freeze–thaw cycle. Powell et al. [11] reported the degradations of some proteins resulted from 4 to 7 freeze–thaw cycles in urinary proteomics.

Furthermore, Klasen et al. [20] found some proteins forming precipitates after storage and thawing. According to the studies of Saetun et al. [23], after overnight storage at −20 °C, urinary proteins may precipitate, and they found that EDTA (5 mM) could reduce the amount of precipitates and pH could influence the type of precipitates. To redissolve the precipitates, effectively shaking of the specimens should be done at room temperature.

4.2.6 pH

Thongboonkerd et al. [24] analyzed adjusted pH urinary samples which were precipitated the proteins by 75 % ethanol. The 2-DE results showed different pH levels did not influence the consistency of individual urinary specimens and the total number of spots. Therefore, they thought it was unnecessary to adjust the pH of urinary samples before gel-based proteome analysis.

4.2.7 Standard Protocol for Urine Collection

Based on previous studies, a standard protocol for urinary collection was recommended by Human Kidney and Urine Proteome Project, HUKPP, and European Urine and Kidney Proteomics, EuroKUP Initiatives from 9 December 2009.

The details were described as follows:

Standard Protocol for Urinary Collection [25]

1. Type of urinary sample
   Midstream of second-morning urine (preferably) or morning random-catch urine, in sterile (preferably) or clean urinary collectors.
2. Pre-treatment and storage
   Centrifuge at 1,000*g*, for 10 min to remove cell debris and casts. Aliquot supernatant avoiding disturbing the pellets at 1.5, 10, or 50 mL (depending on downstream application); Do not overfill the tubes; Store at −80 (preferably) or −20 °C. Record time until freezing (it should be no longer than 3 h).
3. Freezing and thawing
   Avoid freeze–thaw cycles. If thawing and re-freezing occurs, always keep a record of this event.

Notes: http:/www.hukpp.org and www.eurokup.org.

4.3 Urinary Preparation

For urinary proteomics, crude urinary samples are complex including high concentrations of salts, small molecules, and some metabolic wastes [26], and concentration of urinary proteins is too low to be identified. So many sample preparation methods have been applied to concentrate urinary proteins and remove small molecules, such as organic solvent precipitation, ultracentrifugation, dialysis–lyophilization, and ultrafiltration (centrifugal filtration) [26, 27].

4.3.1 Organic Solvent Precipitation

Organic solvent precipitation is a popular method in urinary proteomics. Organic solvent can reduce of permittivity of urine and break hydration shell on the surface of protein molecules, and thus, urinary proteins are gathered and precipitated effectively. Organic solvent precipitation method had several characters. First, this approach can be used to handle larger volumes of urine and takes less time than other methods [27]. Second, it can enrich higher molecular weight proteins species than ultrafiltration [28]. Third, it can effectively precipitate more acidic and hydrophilic proteins than ultracentrifugation [6].

Studies showed types and concentration of organic solvents may play various roles on urinary protein precipitation [14, 27]. Khan et al. [27] employed 2-DE to analyze the urinary proteome precipitated by different organic solvents and found that higher resolution and more protein spots could be obtained using acetonitrile (urine-to-solvent ratio was 1:5). Thongboonkerd et al. [14] made a comprehensive comparison of different organic solvent precipitation methods by 2-DE. They revealed applying 90 or 75 % organic compounds could get greater recovery yield than lower percentage of organic compounds. Ethanol, methanol, or acetone precipitation methods were suggested to obtain more protein spots and higher protein recovery yield in routine or gel-based urinary proteome studies. Moreover, acetonitrile precipitation was suggested for proteinuric urine or a larger volume of urine for its highest number of protein spots but lower protein recovery yield.

4.3.2 Ultracentrifugation

Ultracentrifugation method isolated proteins due to easy sedimentation of high-density protein molecular under ultracentrifugation situation. In 2002, Thongboonkerd et al. [6] analyzed urines from five normal donors by 2-DE and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF). They found ultracentrifugation method could fractionate more basic, hydrophobic, and membrane proteins than organic solvent precipitation approach. But ultracentrifugation uses expensive equipment and acidic proteins are lost, which might limit its application [6].

4.3.3 Dialysis

Another commonly used method is to combine dialysis with lyophilization [29]. Oh et al. [29] reported that dialysis–lyophilization approach was likely to profile the whole urinary proteins on 2-DE and could improve reproducibility and resolution, because it could effectively remove the molecules interfered the profiling of 2-DE. Moreover, Thongboonkerd et al. [14] revealed that this method had great protein recovery yield but showed lowest number of protein spots compared with precipitation, ultracentrifugation, and ultrafiltration method.

4.3.4 Ultrafiltration

As for ultrafiltration method, it uses ultrafiltration membranes to discard small molecules and concentrate urinary protein according to molecular weight difference. Court et al. [30] reported that ultrafiltration method enriched lower molecular

weight proteins than 6 % TCA precipitation by SDS-PAGE gel. Based on ultrafiltration method, Vaezzadeh et al. [31] put forward a one-step sample preparation method. They added urinary sample together with Anti-HSA resin to a Vivaspin 6 spin-filter, which could isolate proteins and remove human serum albumin in one step. It realized sample concentration, purification, and albumin depletion simultaneously. Furthermore, they found that neutral pH (7–8) could achieve both of efficient depletion and high protein recovery.

4.4 Normal Human Urinary Proteomes Analysis

Many researchers had undertaken studies to catalog the normal human urinary proteome. The first study came from Anderson et al. [32] in 1979. They found 250 urinary protein spots by 2-DE. But without high-throughput protein identification approach, it was hard to profile the components of urinary proteome. The development of two ionization methods in MS, matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI), made the precise analysis of biomacromolecule possible [33]. In 2001, Spahr et al. [28] firstly employed LC-MS approach to analyze the human urinary proteome and identified 124 urinary proteins. After that, many groups contributed their efforts to profile a comprehensive normal human urinary proteome. 2-DE and LC-MS were two popular approaches for proteome analysis.

4.4.1 2-DE Approach

In 2002, Thongboonkerd et al. [6] reported their study utilizing acetone precipitation and ultracentrifugation preparation methods. By 2-DE and MALDI-TOF, they identified 47 unique proteins, 28 from acetone precipitation method and 19 from ultracentrifugation method.

In 2004, Oh et al. [29] prepared urinary samples by dialysis–lyophilization and removed albumin using Affi-Gel Blue. They identified 113 urinary proteins on 2-DE by peptide mass fingerprinting with MALDI-TOF-MS analysis. In the same year, Pieper et al. [7] reported a large-scale urinary proteome analysis. First, they fractionated urinary proteins by size exclusion chromatography and collected two fractions, higher than 30-KDa and lower than 30-KDa. Then, they employed immunoaffinity subtraction chromatography to remove albumin and immunoglobulin G from higher than 30-KDa fractions. At last, the two fractions were separated by 2-DE. A total of 1,400 distinct protein spots were found, and 420 spots of these were identified to 150 unique protein.

In 2005, Smith et al. [34] collected 35 urinary samples from 12 donators and extracted the urinary proteins by solid-phase extraction method. By 2-DE and MALDI-TOF/TOF analysis, 48 nonredundant proteins were identified.

In 2006, Khan et al. [27] used ultrafiltration and different organic solvent precipitation method to isolate urinary proteins, and a total of 339 proteins were found with 2-DE separations followed by MALDI-TOF analysis. Zerefos et al. [35] exploited preparative electrophoresis to separated urinary proteins, and by 2-DE and MALDI analysis, 778 protein spots were found and 141 proteins were identified.

4.4.2 LC-MS

In 2002, Pang et al. [36] applied 2DLC-MS method and identified 51 urinary proteins from normal human urinary proteome.

In 2005, Sun et al. [37] applied three approaches to analyze the urinary proteome, 1DE plus 1DLC-MS, direct 1D LC-MS and 2DLC-MS. They identified 226 urinary proteins, 171 proteins of which were identified for the first time. Castagna et al. [38] used hexameric peptide libraries methods to reduce the high-abundant proteins and enrich medium and low abundant ones in urinary proteome. By this method, they identified 383 unique proteins and 251 proteins were not ever found.

In 2008, Lee et al. [39] handled urinary samples by four different approaches: vacuum centrifugation, 90 % ethanol precipitation, microconcentrator, and reverse-phase trapping column. By in-gel digestion and LC-MS analysis, 154, 154, 162 and 148 proteins were identified, respectively, in four preparation methods and 600 proteins were found in total [40].

In 2009, Kim et al. [41] modified isoelectric focusing and asymmetrical flow field-flow fractionation by applying Teflon tubing to connect multilane asymmetrical flow field-flow fractionation (AF4) channel with isoelectric focusing (IEF) channel (prevent the possible protein adsorption by membrane wall of IEF segments). The fractions from IEF were analyzed by LC-MS and 245 urinary proteins were identified.

Urinary protein posttranslational modification was an important issue for urinary proteome analysis. In 2006, Wang et al. [42] utilized concanavalin A to enrich N-linked glycoproteins from normal urinary proteome. By 1DE plus 1DLC-MS and 2DLC-MS, a total of 225 glycoproteins were identified, 150 annotated as glycoproteins by Swiss-Prot and 43 by NetNGlyc 1.0.

4.4.3 High-Resolution MS Analysis

Along with great improvement of mass accuracy of mass spectrometer, new generations of high-resolution MS dramatically increased protein identification for proteomics [43].

In 2006, Jun Adachi et al. [44] reported the first urinary proteome analysis by high-resolution MS. They analyzed in-gel and in-solution digestion urinary samples by LTQ-FTICR and LTQ-Orbitrap. By combining 1,281 proteins from LTQ-FTICR

with 1,055 proteins from LTQ-Orbitrap, a total of 1,543 urinary proteins were obtained from this in-depth study. Gene Ontology (GO) analysis exhibited that membrane proteins occupied nearly half of the annotated proteins. Extracellular proteins were overrepresented and intracellular proteins were underrepresented. However, plasma membrane proteins and lysosome proteins were unexpectedly overrepresented.

In 2010, Goo et al. [45] analyzed the urinary samples from ten female healthy persons by LC coupled with a hybrid linear ion trap–orbitrap mass spectrometer and identified 1,003 urinary proteins. Li et al. [46] used urines from three healthy male donors, and digested peptides were fractionated by two approaches, integrated multidimensional liquid chromatography and Yin-Yang multidimensional liquid chromatography methods. 6,739 unique peptides and 1,310 nonredundant proteins were obtained by two approaches. Furthermore, they did the first large-scale work to profile urinary phosphoproteome and found 45 unique phosphopeptides from 31 phosphoproteins. Most of the phosphorylation sites were on serine residues except for six on threonine and only one on tyrosine residues.

In 2011, Marimuthu et al. [40] reported the first urinary proteome result of high-resolution MS/MS with LTQ-Orbitrap Velos mass spectrometer. They exploited in-gel digestion and LC-MS approach to analyze unfractionated proteins of the pooled urine, as well the glycoproteins after the lectin affinity enrichment. 1,452 proteins were found in unfractionated urine and 617 proteins in glycoproteome. A total of 1,823 proteins were found, and 671 proteins of these proteins were identified in human urine for the first time. Two hundred and sixty-five proteins out of 617 enriched proteins were glycosylated. Forty-four peptides out of 131 peptides identified with protein N-terminus were analyzed to be acetylated.

4.5 Conclusion and Outlook

Following the development of MS technologies, precision proteomics become more and more significant in proteomics. It could not only reveal more proteins secreted in urine but also avoid more errors which lead to misdirected results [50]. Especially, in 2011, Marimuthu et al. [47] published the first urinary proteome study with both of MS and MS/MS at high resolution exhibiting more credible results. With the application of urinary proteome to clinical researches, a larger precision urinary proteome database should be developed, which should be used as a reference for further study.

Another important issue for urinary proteome was high-throughput quantitation. Quantitation of urinary proteins has been proposed, and many approaches were exploited to realize relative and absolute quantitation. In 2013, Nolen et al. [48] applied multiplexed bead-based immunoassays and made absolute quantitation of 211 proteins in healthy urinary samples. However, more than 600 proteins could be identified in only one 1DLC-MS run [49]. Therefore, high-throughput urinary protein quantitation, especially absolute quantitation, still needs more concern.

References

1. Hu S, Loo JA, Wong DT (2006) Human body fluid proteome analysis. Proteomics 6:6326–6353
2. Elkind MS, Tai W, Coates K, Paik MC, Sacco RL (2006) High-sensitivity C-reactive protein, lipoprotein-associated phospholipase A2, and outcome after ischemic stroke. Arch Intern Med 166(19):2073–2080. doi:10.1001/archinte.166.19.2073
3. Rossing K, Mischak H, Parving HH, Christensen PK, Walden M, Hillmann M, Kaiser T (2005) Impact of diabetic nephropathy and angiotensin II receptor blockade on urinary polypeptide patterns. Kidney Int 68(1):193-205. doi:10.1111/j.1523-1755.2005.00394.x
4. Cicenas J, Urban P, Vuaroqueaux V, Labuhn M, Kung W, Wight E, Mayhew M, Eppenberger U, Eppenberger-Castori S (2005) Increased level of phosphorylated akt measured by chemiluminescence-linked immunosorbent assay is a predictor of poor prognosis in primary breast cancer overexpressing ErbB-2. Breast Cancer Res 7(4):R394–R401. doi:10.1186/bcr1015
5. Decramer S, Gonzalez DPA, Breuil B, Mischak H, Monsarrat B, Bascands JL, Schanstra JP (2008) Urine in clinical proteomics. Mol Cell Proteomics 7(10):1850–1862. doi:10.1074/mcp.R800001-MCP200
6. Thongboonkerd V, McLeish KR, Arthur JM, Klein JB (2002) Proteomic analysis of normal human urinary proteins isolated by acetone precipitation or ultracentrifugation. Kidney Int 62 (4):1461–1469. doi:10.1111/j.1523-1755.2002.kid565.x
7. 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 (4):1159–1174. doi:10.1002/pmic.200300661
8. Wu J, Chen YD, Gu W (2010) Urinary proteomics as a novel tool for biomarker discovery in kidney diseases. J Zhejiang Univ Sci B 11(4):227–237. doi:10.1631/jzus.B0900327
9. 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(1):48–61
10. Sun W, Chen Y, Li F, Zhang L, Yang R, Zhang Z, Zheng D, Gao Y (2009) Dynamic urinary proteomic analysis reveals stable proteins to be potential biomarkers. PROTEOMICS—Clinical Applications 3(3):370–382. doi:10.1002/prca.200800061
11. Thongboonkerd V (2007) Practical points in urinary proteomics. J Proteome Res 6 (10):3881–3890. doi:10.1021/pr070328s
12. Bottini PV, Ribeiro AM, Garlipp CR (2002) Electrophoretic pattern of concentrated urine: comparison between 24-hour collection and random samples. Am J Kidney Dis 39(1):E2. doi:10.1053/ajkd.2002.29920
13. Hoorn EJ, Pisitkun T, Zietse R, Gross P, Frokiaer J, Wang NS, Gonzales PA, Star RA, Knepper MA (2005) Prospects for urinary proteomics: exosomes as a source of urinary biomarkers. Nephrology (Carlton) 10(3):283–290. doi:10.1111/j.1440-1797.2005.00387.x
14. Thongboonkerd V, Chutipongtanate S, Kanlaya R (2006) Systematic evaluation of sample preparation methods for gel-based human urinary proteomics: quantity, quality, and variability. J Proteome Res 5(1):183–191. doi:10.1021/pr0502525
15. Lifshitz E, Kramer L (2000) Outpatient urine culture: does collection technique matter? Arch Intern Med 160(16):2537–2540
16. Schaub S, Wilkins J, Weiler T, Sangster K, Rush D, Nickerson P (2004) Urine protein profiling with surface-enhanced laser-desorption/ionization time-of-flight mass spectrometry. Kidney Int 65(1):323–332. doi:10.1111/j.1523-1755.2004.00352.x

17. Havanapan PO, Thongboonkerd V (2009) Are protease inhibitors required for gel-based proteomics of kidney and urine? J Proteome Res 8(6):3109–3117. doi:10.1021/pr900015q
18. Shinada M, Akdeniz A, Panagiotopoulos S, Jerums G, Bach LA (2000) Proteolysis of insulin-like growth factor-binding protein-3 is increased in urine from patients with diabetic nephropathy. J Clin Endocrinol Metab 85(3):1163–1169. doi:10.1210/jcem.85.3.6486
19. 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(11):4173–4181. doi:10.1021/pr070311+
20. Klasen IS, Reichert LJ, de Kat AC, Wetzels JF (1999) Quantitative determination of low and high molecular weight proteins in human urine: influence of temperature and storage time. Clin Chem 45(3):430–432
21. Rosenling T, Slim CL, Christin C, Coulier L, Shi S, Stoop MP, Bosman J, Suits F, Horvatovich PL, Stockhofe-Zurwieden N, Vreeken R, Hankemeier T, van Gool AJ, Luider TM, Bischoff R (2009) The effect of preanalytical factors on stability of the proteome and selected metabolites in cerebrospinal fluid (CSF). J Proteome Res 8(12):5511–5522. doi:10.1021/pr9005876
22. Hsieh SY, Chen RK, Pan YH, Lee HL (2006) Systematical evaluation of the effects of sample collection procedures on low-molecular-weight serum/plasma proteome profiling. Proteomics 6(10):3189–3198. doi:10.1002/pmic.200500535
23. Saetun P, Semangoen T, Thongboonkerd V (2009) Characterizations of urinary sediments precipitated after freezing and their effects on urinary protein and chemical analyses. Am J Physiol Renal Physiol 296(6):F1346–F1354. doi:10.1152/ajprenal.90736.2008
24. Thongboonkerd V, Mungdee S, Chiangjong W (2009) Should urine pH be adjusted prior to gel-based proteome analysis? J Proteome Res 8(6):3206–3211. doi:10.1021/pr900127x
25. http://www.hkupp.org and http://www.eurokup.org (Reprinted)
26. Tantipaiboonwong P, Sinchaikul S, Sriyam S, Phutrakul S, Chen ST (2005) Different techniques for urinary protein analysis of normal and lung cancer patients. Proteomics 5 (4):1140–1149. doi:10.1002/pmic.200401143
27. Khan A, Packer NH (2006) Simple urinary sample preparation for proteomic analysis. J Proteome Res 5(10):2824–2838. doi:10.1021/pr060305y
28. 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(1):93–107. doi:10.1002/1615-9861(200101)1:1<93::AID-PROT93>3.0.CO;2-3
29. 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 (11):3485–3497. doi:10.1002/pmic.200401018
30. Court M, Selevsek N, Matondo M, Allory Y, Garin J, Masselon CD, Domon B (2011) Toward a standardized urine proteome analysis methodology. Proteomics 11(6):1160–1171. doi:10.1002/pmic.201000566
31. 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 (11):6082-6089. doi:10.1021/pr100924s
32. Anderson NG, Anderson NL, Tollaksen SL (1979) Proteins of human urine. I. Concentration and analysis by two-dimensional electrophoresis. Clin Chem 25(7):1199–1210
33. Costello CE (1997) Time, life ... and mass spectrometry. New techniques to address biological questions. Biophys Chem 68(1–3):173–188
34. Smith G, Barratt D, Rowlinson R, Nickson J, Tonge R (2005) Development of a high-throughput method for preparing human urine for two-dimensional electrophoresis. Proteomics 5(9):2315–2318. doi:10.1002/pmic.200401267
35. Zerefos PG, Vougas K, Dimitraki P, Kossida S, Petrolekas A, Stravodimos K, Giannopoulos A, Fountoulakis M, Vlahou A (2006) Characterization of the human urine proteome by

preparative electrophoresis in combination with 2-DE. Proteomics 6(15):4346–4355. doi:10.1002/pmic.200500671
36. Pang JX, Ginanni N, Dongre AR, Hefta SA, Opitek GJ (2002) Biomarker discovery in urine by proteomics. J Proteome Res 1(2):161–169
37. Sun W, Li F, Wu S, Wang X, Zheng D, Wang J, Gao Y (2005) Human urine proteome analysis by three separation approaches. Proteomics 5(18):4994–5001. doi:10.1002/pmic.200401334
38. Castagna A, Cecconi D, Sennels L, Rappsilber J, Guerrier L, Fortis F, Boschetti E, Lomas L, Righetti PG (2005) Exploring the hidden human urinary proteome via ligand library beads. J Proteome Res 4(6):1917–1930. doi:10.1021/pr050153r
39. Lee RS, Monigatti F, Briscoe AC, Waldon Z, Freeman MR, Steen H (2008) Optimizing sample handling for urinary proteomics. J Proteome Res 7(9):4022–4030. doi:10.1021/pr800301h
40. 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(6):2734–2743. doi:10.1021/pr2003038
41. Kim KH, Moon MH (2009) High speed two-dimensional protein separation without gel by isoelectric focusing-asymmetrical flow field flow fractionation: application to urinary proteome. J Proteome Res 8(9):4272–4278. doi:10.1021/pr900363s
42. Wang L, Li F, Sun W, Wu S, Wang X, Zhang L, Zheng D, Wang J, Gao Y (2006) Concanavalin A-captured glycoproteins in healthy human urine. Mol Cell Proteomics 5(3):560–562. doi:10.1074/mcp.D500013-MCP200
43. Olsen JV, de Godoy LM, Li G, Macek B, Mortensen P, Pesch R, Makarov A, Lange O, Horning S, Mann M (2005) Parts per million mass accuracy on an Orbitrap mass spectrometer via lock mass injection into a C-trap. Mol Cell Proteomics 4(12):2010–2021. doi:10.1074/mcp.T500030-MCP200
44. 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(9):R80. doi:10.1186/gb-2006-7-9-R80
45. Goo YA, Tsai YS, Liu AY, Goodlett DR, Yang CC (2010) Urinary proteomics evaluation in interstitial cystitis/painful bladder syndrome: a pilot study. Int Braz J Urol 36(4):464–478, 478–479, 479
46. Li QR, Fan KX, Li RX, Dai J, Wu CC, Zhao SL, Wu JR, Shieh CH, Zeng R (2010) A comprehensive and non-prefractionation on the protein level approach for the human urinary proteome: touching phosphorylation in urine. Rapid Commun Mass Spectrom 24(6):823–832. doi:10.1002/rcm.4441
47. Mann M, Kelleher NL (2008) Precision proteomics: the case for high resolution and high mass accuracy. Proc Natl Acad Sci U S A 105(47):18132–18138. doi:10.1073/pnas.0800788105
48. Nolen BM, Orlichenko LS, Marrangoni A, Velikokhatnaya L, Prosser D, Grizzle WE, Ho K, Jenkins FJ, Bovbjerg DH, Lokshin AE (2013) An extensive targeted proteomic analysis of disease-related protein biomarkers in urine from healthy donors. PLoS One 8(5):e63368. doi:10.1371/journal.pone.0063368
49. Nagaraj N, Mann M (2011) Quantitative analysis of the intra- and inter-individual variability of the normal urinary proteome. J Proteome Res 10(2):637–645. doi:10.1021/pr100835s