

# Chapter 2

## Human Urine Proteome: A Powerful Source for Clinical Research



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**Abstract** As noninvasive and easily available biological fluid, urine is becoming an ideal sample for clinical disease biomarker 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 due to 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

### 2.1 Introduction

Body fluids have been regarded as the significant source of biomarkers, which could be used for the early diagnosis and state forecast of disease in clinical (Hu et al. 2006; Elkind et al. 2006; Rossing et al. 2005; Cicens et al. 2005). As a body fluid, urine is an important resource of disease biomarker discovery.

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 (Decramer et al. 2008). Approximately 30% of urinary proteins originate from the plasma proteins, whereas 70% comes from the kidney and the urinary tract (Thongboonkerd et al. 2002a). Therefore the urinary proteome might supply important biomarkers directly reflecting the functions of the kidney and related organs (Wu et al. 2010). As one of the most attractive sources for biomarker detecting, urine has showed several advantages: (1) urine can accumulate changes from the body; most of the waste in the blood that reaches the urine can tolerate a much higher degree of change; thus, biomarkers in urine are more likely

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to be magnified and detectable than in blood (Gao 2013); (2) it is easy to be collected in large amount and noninvasive way; (3) urine samples are less complex than plasma and carry many proteins, peptides, and amino acids that have not been discovered in plasma (Anderson et al. 1979a). Therefore, many researchers did their best to have a deeper understanding of urine 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 urine proteome analysis in recent years, which may be helpful for further studies.

## 2.2 Collection and Storage

### 2.2.1 *The Types of Urine*

In clinic, many kinds of urine samples have been used according to the different examination. However, many factors may influence the components of urinary proteome (Sun et al. 2009), including daily activities, physiological variations, and environmental factors such as temperature and pH. Therefore, several types of urine samples have been analyzed by proteomic approach, including first morning urine, 24-h urine, second morning urine, random urine, the urine collected after drinking a large amount of water or after drinking coffee, etc.

24-h urine can show the excretion of urinary proteins within a day (Thongboonkerd 2007). 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 (Bottini et al. 2002). Concerning first morning urine, it can't exhibit urine "diurnal variation" (different time-points' variation in all day) (Thongboonkerd 2007). And Hoorn et al. (2005) reported that first morning urine may have bacterial contamination due to the long residence time in the bladder.

Sun et al. (2009) 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. Other studies (Thongboonkerd et al. 2006; Peerapen et al. 2017) compared four different time-point 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 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, par-

ticularly for women (Lifshitz and Kramer 2000). Schaub et al. (2004) 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. But for female samples, first-void urine emerged three specific SELDI peptide peaks after a 3-day storage compared with midstream.

### ***2.2.2 Protease Inhibitor***

Protease inhibitors had been initially suggested to be used to prevent proteolysis of clinical biological fluids, which was caused by endogenous proteases (Havanapan and Thongboonkerd 2009). However, whether protease inhibitors are necessary for the studies of urinary proteomics is debatable. Shinada et al. (2000) incubated 30 kDa nonglycosylated [<sup>125</sup>I] IGFBP-3 with urine samples and found IGFBP-3 proteolysis by SDS-PAGE analysis. But Havanapan and Thongboonkerd (2009) studied the effect of protease inhibitors cocktail on midstream random urine specimens and found that no observable qualitative and quantitative changes by two-dimensional gel electrophoresis (2-DE) analysis. Thongboonkerd (2007) suggested protease inhibitors were unnecessary for the studies of nonproteinuric urine because there were lower amounts of proteases in urine than in plasma, cells, or tissues. In 2010, Maryam Afkarian et al. demonstrated that absence of protease inhibitors did not affect the identification of the high confidence proteins (Afkarian et al. 2010). In the same year, Kania et al. (2010) investigated the albumin fragmentation by using the method of nephelometry, HPLC, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Albumin concentration measured by HPLC was most dramatically affected, with near-complete loss of albumin-sized material within 1 h of incubation at pH 2.3–2.5. Their results showed that urinary albumin digestion occurred in a manner consistent with the activity of endogenous urinary proteases. And they recommended that the adjustment to neutral pH or addition of protease inhibitors may be useful techniques for urine sample preservation.

### ***2.2.3 Preservatives***

During the storage, urine samples might have bacterial overgrowth, which could change the urinary proteome (Thongboonkerd and Saetun 2007). Therefore preservatives were recommended to prevent the bacterial overgrowth after collection (Thongboonkerd and Saetun 2007). Thongboonkerd and Saetun (2007) studied the effects of the addition of either sodium azide (NaN<sub>3</sub>) or boric acid on bacterial overgrowth in pooled urine from five healthy individuals. They found the addition of NaN<sub>3</sub> 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  $\text{NaN}_3$  to one void random urine specimens and addition of 200 mM boric acid or 10 mM  $\text{NaN}_3$  to 24-h urine collection.

Recently, Remer et al. (2014) examined the long-term stability and validity of analyte concentrations of 21 clinical biochemistry parameters (creatinine, urea, iodine, nitrogen, sodium, uric acid, and so on) in 24-h urine samples stored at  $-22\text{ }^\circ\text{C}$  and preservative-free. They suggested high long-term (>10 years) stability and measurement validity for numerous clinical chemistry parameters when stored at  $-22\text{ }^\circ\text{C}$  without any urine preservative. Porter IA et al. (Porter and Brodie 1969) reported that boric acid adequately could preserve urine specimens for up to 24 h. They compared the results of bacteriological culture and microscopic examination of urine samples transported over a distance by the dip-inoculum transport medium, icebox, and boric acid preservation with “natural” urine specimens, and the results showed that the “natural” urine gives satisfactory preservation.

#### **2.2.4 Storage Temperature**

Appropriate storage temperature could decrease degradation of urine proteins to some extent. In 1999, Klasek et al. (1999) analyzed changes of albumin concentrations after urine samples were stored at  $4\text{ }^\circ\text{C}$ ,  $-20\text{ }^\circ\text{C}$ , and  $-70\text{ }^\circ\text{C}$ . They found that if samples could be analyzed in 4 weeks after collected, the best storage temperature was  $4\text{ }^\circ\text{C}$ . And they recommended  $-70\text{ }^\circ\text{C}$  is used as storage temperature for longer storage. They did not suggest storage temperature  $-20\text{ }^\circ\text{C}$  because they found the IgG concentrations decreased after 1-week storage at  $-20\text{ }^\circ\text{C}$ . In 2007, Thongboonkerd and Saetun (2007) reported that to prevent the bacterial overgrowth, uncentrifuged urine samples without preservatives should be kept no longer than 20 h at  $4\text{ }^\circ\text{C}$ . Without any preservative, urine samples should not be stored at room temperature for longer than 8 h. In 2008, Lee et al. (2008a) reported that peptide and urine proteins are very stable at room temperature up to 24 h by SDS-PAGE analysis. In 2011, Molina et al. (2011) evaluated the impact of temperature after urine collection and before freezing at  $-80\text{ }^\circ\text{C}$ . In their study, urines were kept at room temperature (RT) for 1 h and then stored at  $+4\text{ }^\circ\text{C}$  or at RT for another 7 h, and no quantitative difference was found by 2D-GE. But Hindman et al. (1976) reported that urine stored at room temperature for more than 2 h showed an overgrowth of microorganisms.

#### **2.2.5 Freeze-Thaw Cycle**

Previous studies showed freeze-thaw cycles could influence the components of body fluid proteome, including serum/plasma and cerebrospinal fluid (Rosenling et al. 2009; Hsieh et al. 2006). Studies also showed that when urine samples were stored at low temperature, researchers should avoid freeze-thaw cycles

(Thongboonkerd 2007). Schaub et al. (2004) analyzed first-void and midstream urine specimens from three females and three males by SELDI-TOF MS. Results indicated that after 1 to 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. Besides, some studies (Lee et al. 2008a; Powell et al. 2006; Bao 2009) reported the degradations of some proteins resulted from 4 to 7 freeze-thaw cycles in urinary proteomics. Furthermore, Klasen et al. (1999) found some proteins forming precipitates after storage and thawing. According to the studies of Saetun et al. (2009), after overnight storage at  $-20^{\circ}\text{C}$ , 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.

### 2.2.6 pH

Thongboonkerd et al. (2009) analyzed pH adjusted urine samples which were precipitated by 75% ethanol. The 2-DE results showed that different pH levels did not influence the consistency of individual urine specimens and the total number of spots. Therefore, they thought it was unnecessary to adjust the pH of urine samples before gel-based proteome analysis.

### 2.2.7 *Standard Protocol for Urine Collection*

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

The details were described as follows:

**Standard Protocol for Urine Collection (<http://www.hkupp.org> and <http://www.Eurokup.org>)**

#### 1. Type of urine sample

Midstream of second morning urine (preferably) or morning random-catch urine, in sterile (preferably) or clean urine collectors.

#### 2. Pretreatment and storage

Centrifuge at 1000 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  $-80^{\circ}\text{C}$  (preferably) or  $-20^{\circ}\text{C}$ . 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.hkupp.org> and <http://www.Eurokup.org>.

## 2.3 Urine Preserved on Membrane

In 2014, Jia et al. (2014) proposed a method for adsorbing urinary proteins onto a polyvinylidene difluoride (PVDF) membrane named Urimem. In their study, urine samples were filtered through the membrane, and urinary proteins were adsorbed on it. The proteins on the membrane were dried and could be stored in a vacuum bag to keep the preservation of protein pattern faithfully. The loading capacity of the PVDF membrane was tested using SDS-PAGE analysis. The results showed that most of the proteins were adsorbed on the membrane during the first adsorption. The method has showed several advantages due to simplicity, low cost, nonrequirement of organic solvents, and minimal sample handling. Furthermore, proteins on the membrane could be even stored at room temperature for more than 2 weeks.

Based on Jia's work, similar as Jia et al. (2014), Somchai Chutipongtanate et al. (2015) developed a simple rapid method of urine preparation named syringe-push membrane absorption (SPMA) in 2015, which combined 5-mL medical syringe with protein-absorbable membrane. In the study, they found nitrocellulose to be the most suitable membrane to combine with SPMA, which provided the greatest quality of proteome profile by 2-DE analysis. Comparing with three current methods of urine preparation (i.e., ultrafiltration, dialysis/lyophilization, and precipitation), nitrocellulose-SPMA had better working performance due to acceptable recovery yield, less workload, short working time, high accessibility, and low unit cost. In addition, protein absorbed on nitrocellulose harvested from the SPMA procedure could be stored as a dried membrane at room temperature for at least 1 month without protein degradation or modification.

For the protein recovery from the membrane, Qin and Gao (2015) developed a method to elute the urinary proteins from nitrocellulose membrane with heating. They raised the temperature to reduce the intense vortexing time, and gentle rotating was kept while precipitation to prevent nitrocellulose reformation. By SDS-PAGE and LC-MS/MS, the urinary proteins prepared by heating elution procedure showed no degradation of proteins.

## 2.4 Urine Preparation

For urinary proteomics, crude urine samples are complex including high concentrations of salts, small molecules, and some metabolic wastes (Tantipaiboonwong et al. 2005a), and concentration of urinary proteins are too low to be identified. So

many sample preparation methods have been applied to concentrate urine proteins and remove small molecules, such as organic solvent precipitation, ultracentrifugation, dialysis-lyophilization, and ultrafiltration (centrifugal filtration) (Tantipaiboonwong et al. 2005a; Khan and Packer 2006).

### 2.4.1 Organic Solvent Precipitation

Organic solvent precipitation is a popular method in urinary proteomics. Organic solvent can reduce permittivity of urine and break hydration shell on the surface of protein molecules, thus urine proteins gathered and are 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 (Khan and Packer 2006). Second, it can enrich higher molecular weight protein species than ultrafiltration (Saetun et al. 2009). Third, it can effectively precipitate more acidic and hydrophilic proteins than ultracentrifugation (Thongboonkerd et al. 2002b).

Studies showed types and concentration of organic solvents may play various roles on urinary protein precipitation (Lifshitz and Kramer 2000; Khan and Packer 2006). Khan and Packer (2006) 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). Tantipaiboonwong et al. (2005a) mentioned that addition of trichloroacetic acid as well as trifluoro acid could increase protein yield. Thongboonkerd et al. (2006) 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. Besides, Simpson and Beynon (Simpson and Beynon 2010) found that acetone precipitation may lead to selective modification of peptides, predominantly in the peptides whose second amino acid is glycine residue, which might generate a relatively stable derivative. However, Maryam Afkarian et al. (2010) reported that protein extraction by methanol precipitation would lead to the highest protein yields and the most reproducible spectra.

According to previous studies, disease state and the physicochemical property of urine sample would also affect protein extraction by different solvents (Olszowy and Buszewski 2014). In 2013, Crowell et al. (2013) provided an in-depth characterization of protein recovery through acetone precipitation. They increased the ionic strength of the solution by adding 1–30 Mm NaCl into acetone (50–80%), which dramatically improved the precipitation efficiency of individual proteins, and proteome mixtures (ca. 80–100% yield).

### 2.4.2 *Ultracentrifugation*

Ultracentrifugation is a common method for separating proteins due to easy sedimentation of high-density protein molecular under ultracentrifugation situation. In 2002, Thongboonkerd et al. (2002b) 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 (Thongboonkerd et al. 2002b).

### 2.4.3 *Dialysis*

Another commonly used method is to combine dialysis with lyophilization (Oh et al. 2004). Oh et al. (2004) reported that dialysis-lyophilization approach was likely to profile the whole urine proteins on 2-DE and could improve reproducibility and resolution, because it could effectively remove the molecules that interfered the profiling of 2-DE. Moreover, Thongboonkerd et al. (2006) revealed that this method had great protein recovery yield but showed lowest number of protein spots compared with precipitation, ultracentrifugation, and ultrafiltration method.

### 2.4.4 *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. (2011) reported that ultrafiltration method enriched lower molecular weight proteins than 6% TCA precipitation by SDS-PAGE gel. Based on ultrafiltration method, Vaezzadeh et al. (2010) put forward a one-step sample preparation method. They added urine 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 efficient depletion and high protein recovery. In 2005, Payungsak Tantipaiboonwong et al. (2005b) reported that the sequential preparation of urinary proteins by gel filtration and ultrafiltration obtained the highest number of protein spots on 2D gels and retained the most urinary proteins.

Recently Sebastian T. Berger et al. (2015) described a 96-well plate compatible membrane-based proteomic sample processing method. In their study, a large-pore hydrophobic PVDF membrane was used to efficiently adsorb proteins, resulting in fast liquid transfer through the membrane and significantly reduced sample process-

ing times. Finally, they identified 819 proteins in only 150  $\mu\text{L}$  urine sample by using a 1 h gradient on TripleTOF 5600 + . This method not only was high-throughput and very fast but also could prepare peptide samples by using urine sample directly without protein extraction. Besides, Yanbao Yu et al. (2017) described a FASP method adapted to 96-well filter plates, named 96 FASP, which could also prepare peptide samples by using urine sample directly. In this method,  $\sim 10 \mu\text{g}$  of total urinary protein was reduced, alkylated, and digested in 96-well filter plates directly, resulting in 700–900 protein identification by Q Exactive. The method was suitable for high-throughput quantitative clinical proteomics.

## 2.5 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. (1979b) in 1979. They found 250 urine 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 (Costello 1997). In 2001 Spahr et al. (2001) 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.

### 2.5.1 2-DE Approach

In 2002 Thongboonkerd et al. (2002b) 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. (2004) prepared urine 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. (2004) reported a large-scale urinary proteome analysis. First, they fractionated urine 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. Total 1400 distinct protein spots were found and 420 spots of these were identified to 150 unique protein.

In 2005, Smith et al. (2005) collected 35 urine samples from 12 donors, 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 and Packer (2006) 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. (2006) exploited preparative electrophoresis to separate urinary proteins by 2-DE and MALDI analysis; 778 protein spots were found and 141 proteins were identified.

### 2.5.2 LC-MS

In 2002, Pang et al. (2002) applied 2D LC-MS method and identified 51 urine proteins from normal human urine proteome.

In 2005, Sun et al. (2005) applied three approaches to analyze the urinary proteome, 1DE plus 1D LC-MS, direct 1D LC-MS, and 2D LC-MS. They identified 226 urinary proteins, 171 proteins of which were identified for the first time. Castagna et al. (2005) 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. (2008b) handled urine 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 (Marimuthu et al. 2011).

In 2009, Kim and Moon (2009) 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). 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. (2006) utilized concanavalin A to enrich N-linked glycoproteins from normal urinary proteome. By 1DE plus 1DLC-MS and 2DLC-MS, total 225 glycoproteins were identified, 150 annotated as glycoproteins by Swiss-Prot and 43 by NetNGlyc 1.0.

### 2.5.3 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 (Olsen et al. 2005).

In 2006, Jun Adachi et al. (2006) 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 1281 proteins from LTQ-FTICR with 1055 proteins from LTQ-Orbitrap, total 1543 urine proteins were obtained from this in-depth study. Gene ontology (GO) analysis showed that membrane proteins occupy nearly half of the annotated proteins. Extracellular proteins were over-represented and intracellular proteins were underrepresented. However, plasma membrane proteins and lysosome proteins were unexpectedly overrepresented.

In 2010, Goo et al. (2010) analyzed the urine samples from ten female healthy persons by LC coupled with a hybrid linear ion trap-orbitrap mass spectrometer and identified 1003 urinary proteins. Li et al. (2010) 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. 6739 unique peptides and 1310 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. (2011) 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 as the glycoproteins after the lectin affinity enrichment. 1452 proteins were found in unfractionated urine and 617 proteins in glycoproteome. Total 1823 proteins were found, and 671 proteins of these proteins were identified in human urine for the first time. 265 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.

In 2013, Zheng et al. (2013) performed a proteomic analysis of urine samples from pregnant and nonpregnant patients using SDS-PAGE and LC-MS/MS. In total, 2579 proteins were identified, including 1408 from the urine of pregnant volunteers and 1985 from the nonpregnant group. Total 1023 proteins were not reported in previous studies.

In 2017, Zhao et al. (2018) presented an in-depth analysis of the urinary proteome based on different separation strategies, including direct 1D LC/MS/MS, 2D LC/MS/MS, and gel-eluted liquid fraction entrapment electrophoresis/liquid-phase isoelectric focusing followed by 1D LC/MS/MS. By combining 799 proteins from 1D LC/MS/MS with 2362 proteins identified in 2D analysis and 2924 proteins from 3D analysis, total of 6085 proteins were identified in healthy urine, of which 2001 had not been reported previous. The protein functional analysis showed extracellular proteins and plasma membrane proteins were enriched in 1D analysis, proteins identified in 2D analysis were enriched in intracellular proteins, and proteins in 3D analysis were most in the cytoplasm and nucleus. Moreover, by mapping the urine protein to Human protein Atlas, the tissue distribution of normal urinary protein is also provided. The urinary proteome distributes across 44 tissues; among them, the brain is the tissue with the highest level of both protein and mRNA expression, and

other tissues with more highly expressed proteins were mostly digestive organs, such as the colon and stomach.

Recently Zhao et al. (2015) reported a comprehensive comparison of five body fluids, including urine, plasma, saliva, cerebrospinal fluid, and amniotic fluid using 2D LC/MS/MS approach.

A total of 4717 proteins were identified, and 564 proteins were shared among the five body fluids, with common functions in the coagulation/prothrombin system and inflammatory response. A total of 36.7% of the proteins were detected in only one body fluid and were closely related to their adjacent tissues by function. The functional analysis of the remaining 2986 proteins showed that similar functions might be shared among different body fluids, which highlighted intimate connection in the body. Above results indicated that body fluids might reflect the diverse functions of the whole body rather than the characteristics of their adjacent tissues.

## 2.6 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 (Mann and Kelleher 2008). Especially, in 2011 Marimuthu et al. (2011) 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 urine proteins has been proposed, and many approaches were exploited to realize relative and absolute quantitation. In 2013, Nolen et al. (2013) applied multiplexed bead-based immunoassays and made absolute quantitation of 211 proteins in healthy urine samples. However, more than 600 proteins could be identified in only one IDLC-MS run (Nagaraj and Mann 2011). Therefore, high-throughput urinary protein quantitation, especially absolute quantitation, still needs more concern.

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