ORIGINAL ARTICLE

Assessment of Different Urinary Protein Precipitation Methods for Protein Profiling by Mass Spectrometry

Srinivasa Krishnappa[1], Yalpi Karthik[2], Manjula Shantaram[1,2*]

[1]Department of Studies and Research in Biochemistry, Mangalore University, Jnana Kaveri, Chikka Aluvara, Kodagu, Karnataka, India, [2]Department of Studies and Research in Microbiology, Mangalore University, Jnana Kaveri Post Graduate Centre, Mangalore, Karnataka, India

Abstract

Aim: Urine has become one among the foremost engaging bio-fluids in clinical proteomics because is to often obtained non-invasively in massive quantities and is stable compared to other bio-fluids. The study is mainly focused to determine the best method for urinary protein extraction. **Materials and Methods:** Urinary proteins were precipitated using; chloroform/methanol, acetone, and TCA/acetone solvents followed by liquid chromatography–mass spectrometry (LC-MS) analysis. **Results:** In the present study, we have tested three different methods for urine protein precipitation and qualitatively evaluated by LC-MS-analysis. The protein samples were prepared using acetone, TCA/acetone, and M/C precipitation showed 51, 86, and 114 proteins, respectively. Of the percentage of identified proteins by mass spectrometric analysis revealed that about 17.6% of proteins were found in all the three methods. The highest percentage of shared proteins was observed between acetone and M/C precipitation (69%), followed by 63.8% and 52.7% in TCA/acetone and M/C and TCA/acetone precipitation methods, respectively. By contrast, M/C, TCA/acetone, and acetone precipitations showed 26%, 11.7%, and 12.4% of the unique proteins excluding shared proteins, respectively. The comparison between two different methods, acetone, and M/C, showed the highest percentage of shared protein as 69%, TCA/acetone and M/C precipitation showed 63.8%, and acetone and TCA/acetone precipitation showed 52.7%. M/C, TCA/acetone, and acetone precipitations showed 26%, 11.7%, and 12.4% of the unique proteins excluding shared proteins, respectively. **Conclusion:** This study contributes to establish a standard procedure in urine proteomics. Using urine biomarkers, it can be widely used in urine proteomics not only for diagnosis but also in basic biomedical research, such as physiology and pharmacology.

Key words: Urine proteomics, Clinical diagnosis, Mass spectrometry, Physiology, Pharmacology, Chromatography

INTRODUCTION

The protein analysis of a complete cell and description of post-translationally modified proteins is referred as "proteomics."[1] Urine has become one amongst the foremost engaging bio-fluids in clinical proteomics because it is often obtained non-invasively in massive quantities and is stable compared to other bio-fluids. Urine components embody soluble and insoluble proteins, salts, small molecules, cells and cell debris, extracellular vesicles, and nucleic acids.[2]

The efficacy of urine proteomics has been documented greatly in every year and it has shown different biological processes in the body.[3] The benefit of urine examination is to identify physiological changes and ailments in a person. Blood examination reveals internal surroundings evidently; but, the sampling itself is invasive and repeating many times throughout the study is quite hard.[4] Numerous studies on urinary proteome have resulted as valuable evidence in medical fields.[5] Recently, urine proteomics is actively performed to investigate biomarkers to diagnose various diseases.[6] The standard and size of liquid chromatography–mass spectrometry (LC-MS)/MS analysis data are the most important aspects in urine proteomics. These variables are impacted by the urine protein sample

Address for correspondence:
Manjula Shantaram, Department of Studies and Research in Biochemistry, Mangalore University, Jnana Kaveri, Chikka Aluvara, Kodagu-571232, Karnataka, India.
E-mail: manjula59@gmail.com

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condition as well as the mass spectroscopy and software system used in the study.[7,8]

Proteins are usually isolated from fresh or frozen urine samples using numerous strategies counting on aim of the study. Sample preparation must be reliable for proteomic analysis that the sample should contain more protein concentration and free from other unnecessary elements such as salt, and nucleic acids.[9,10] Precipitation is the most used method to concentrate and fractionate specific protein in biological fluids of an individual.[11,12]

The study used different protein precipitation methods to investigate protein concentration of normal urine samples. Among the valuable sample materials for biomarker discovery of urine assortment is easy, non-invasive, and quantity of sample is comparatively rich compared to different body fluids which holds whole biological data of the body of a person.[13]

The protein study results obtained by precipitation andliquid chromatography-mass spectrometry methods will help to choose the most effective strategies for analyzing changes in the clinical treatments and also in the documentation of biological markers for diseases and to study the mechanism of action of the drug.[14]

MATERIALS AND METHODS

Preparation of samples and proteins

Urine samples from healthy individuals were taken in 50 mL tubes and were divided in three 5 mL tubes and kept at -20°C until needed. Within 6 months of freezing, the urine was used for examination. Before usage, the samples which are kept for freezing were thawed for 10 min at 37°C. To eliminate freeze and thaw cycles, all the frozen urine was thawed and used for analysis.

Study design for each precipitation method

From the original stock, urine was divided in into three tubes (500 µL in each). One tube was used for the initial excretory product protein assay and the other two were used for excretory product protein precipitation. Enzyme digestion and amide purification were performed on one of each of the precipitated samples. After that, it was examined using LC-MS/MS, and the results were utilized to identify proteins. The other sample was utilized for a protein assay to assess for precipitated protein recovery.

Precipitation methods for urine protein preparation

Acetone precipitation

Experiments were carried out at a temperature of 40°C. To four volume of ice-cold acetone one volume of isolated protein sample with 20 mM Dithiothreitol (DTT) was added. The mixture was vortexed before being incubated for 1 h at -20°C. The samples were then centrifuged for 15 min at 40°C at 10000× *g*. The pellet was air dried after the supernatant was discarded.[14]

Methanol/chloroform precipitation

125 µL of chloroform, 500 µL of sample, and the same amount of 100% methanol were added and thoroughly mixed for 5 min. For 15 min, the sample was centrifuged at 12,000× *g*. Pipette was used to extract the supernatant without adhering the interface layer (protein fraction). After that, 500 µL of 100% methanol were carefully mixed into the sample for 5 min. At 25°C, the material was centrifuged at 12,000× *g* for 15 min. The particle was air-dried after the supernatant was discarded. In 200 µL of 8 M urea/50 mMTris-HCl, all of the protein pellets were dissolved at pH 8.0.[14]

10% Trichloroacetic acid (TCA)/acetone precipitation

Tests were carried out at a temperature of 4°C. One volume of protein sample was vortexed with eight volumes of ice-cold acetone. After that, 20% trichloroacetic acid (TCA) was added equal to protein sample and mixed well before being incubated at -20°C for 1 h. After centrifuging the samples at 12,000× *g* for 15 min at 4°C, 0.5 mL ice-cold acetone containing 20mM DTT was added, and the combination was spun at 12,000× *g* for another 15 min at 4°C. Finally, the particle was air dried after the supernatant was removed.[11]

LC-MS analysis

QExactive plus (Thermo Fisher Scientific) online was used in conjunction with a nano-flow HPLC system with a trap column (2 cm × 75 m Acclaim Pepmap 100 column) and a separation column (12.5 cm × 75 m NTCC-360) for mass qualitative analysis. Solution A contained 0.1% FA; solution B had 0.1% FA and 99.9% acetonitrile. Following purification, 500 ng of tryptic peptides were injected onto an analytical column and eluted at a flow rate of 300 nL/min across a 120-min linear gradient of 2% B to 35% B. MS and MS/MS scan ranges of 350–1800 m/z and 200–2000 m/z, respectively, are available. The mass spectrometer was operated in positive mode.

RESULTS

Urine protein extraction is best accomplished using a methanol/chloroform precipitation technique

Minor differences between control and experimental groups were studied in comparative proteomic studies whichare typically important; proper sample preparation is necessary for attaining consistent, reproducible, and significant data.[15]

Following the study plan outlined above, we analyzed the precipitated urine protein and recovery rate to evaluate the capacity of the three distinct types of precipitation methods [Figure 1]. According to the previous publications, to achieve sensitivity and high accuracy in LC-MS/MS analysis, the rate of protein recovery from precipitated urine was one of the most significant requirements. It can identify not only the major proteins in the samples, the trivial ones. We examined the recovery rate of precipitated protein in each precipitation sample.

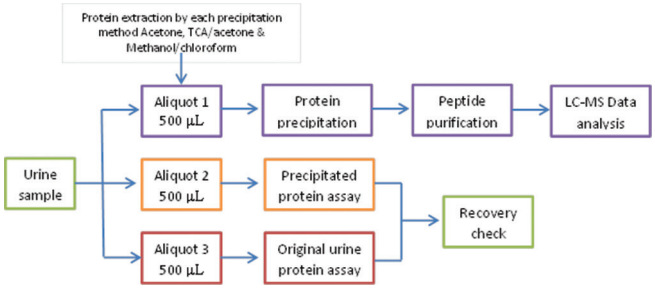

Figure 1: The design of study for the characterization and evaluation of the performance in each precipitation. Crude urine was collected in the container. The 500 µL aliquots of urine samples were used for the investigation of precipitated urine protein recovery and protein identification, respectively.

Using LC-MS/MS analysis, we determined the difference in the quantity of proteins generated by three distinct types of precipitation. Acetone, TCA/acetone, and M/C precipitation produced samples that contained 51, 86, and 114 proteins, respectively. The number of proteins in acetone precipitation was much lower than in the other two precipitations. About 90% of M/C precipitation samples had greater values than the other two precipitations.

The LC-MS results of sample 1 (S1) showed that Figure 2a-c are eluting relatively similar molecular weight proteins 122Da, 104Da, and 144Da and unlike eluted proteins in Figure 2a acetone precipitation method shown 226Da in comparison with Figure 2b TCA/acetone and Figure 2c methanol/chloroform precipitation methods at elution time 0.7 min, respectively. Figure 3a-c showed that 122Da and 104Da are similar proteins at elution time 0.8 min. Figure 4a-c showed 362Da, 475Da, and 476Da at 3.3 min. Figure 5a-c showed 362Da, 365Da, and 475Da at 5.6 min. Figure 6a-c showed all similar proteins such as 301Da, 365Da, 366Da, and 475Da at 5.7 min. Figure 7a-c showed that 301Da, 315Da, 425Da, and 475Da are similar in all three methods and 476Da and 588Da are unique at 5.9 min. Figure 8a-c showed that 301Da, 362Da, 413Da, 475Da, and 588Da are similar and 563Da, 717Da, 689Da, and 690Da are unique at 6.6 min.

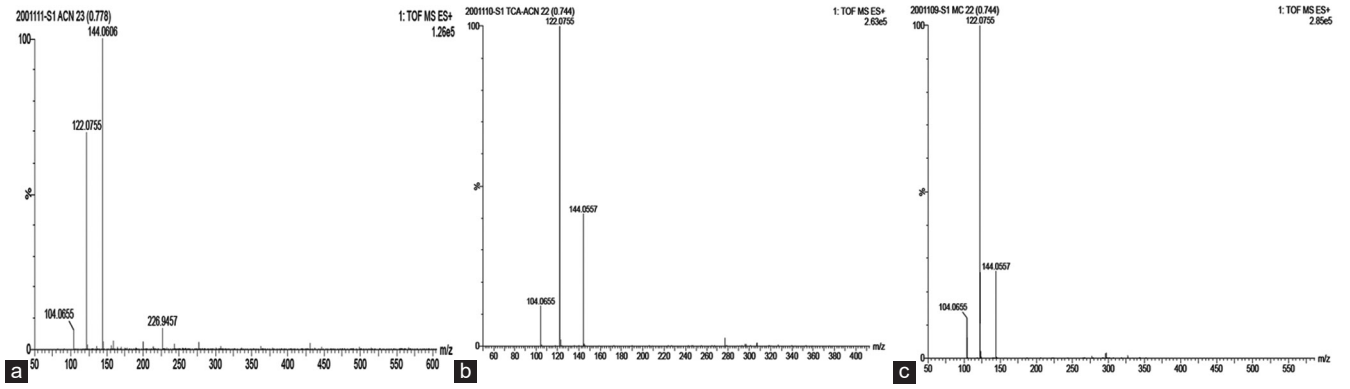

Figure 2: Elution time at 0.7 min of Liquid chromatography – Mass spectrophotometer (a) Acetone precipitation, (b) Trichloro acetic acid and (c) Methanol/chloroform precipitation methods respectively.

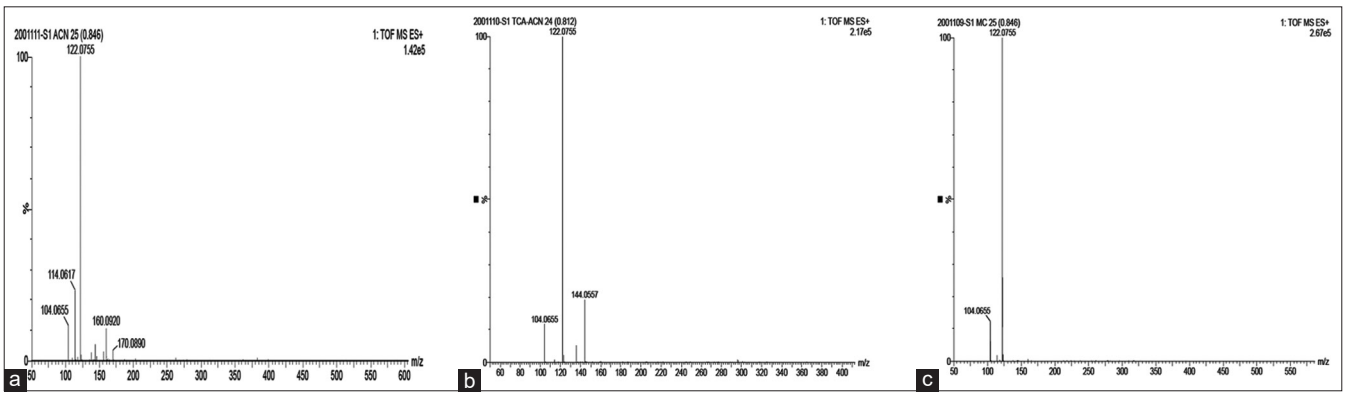

Figure 3: Elution time at 0.8 min of Liquid chromatography – Mass spectrophotometer (a) Acetone precipitation, (b) trichloro acetic acid, and (c) methanol/chloroform precipitation methods, respectively

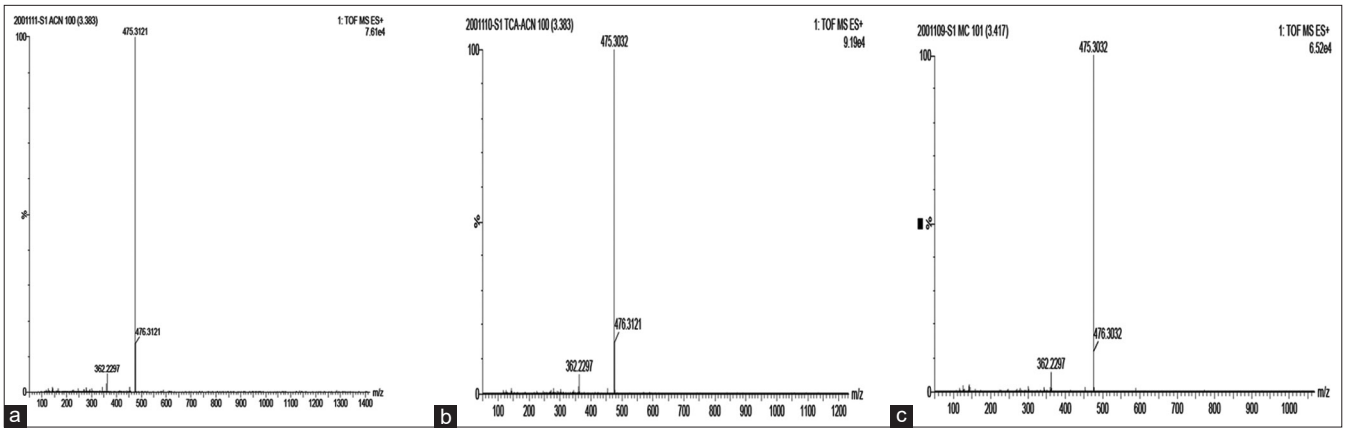

Figure 4: Elution time at 3.4 min of Liquid chromatography – Mass spectrophotometer (a) acetone precipitation, (b) trichloro acetic acid, and (c) methanol/chloroform precipitation methods, respectively

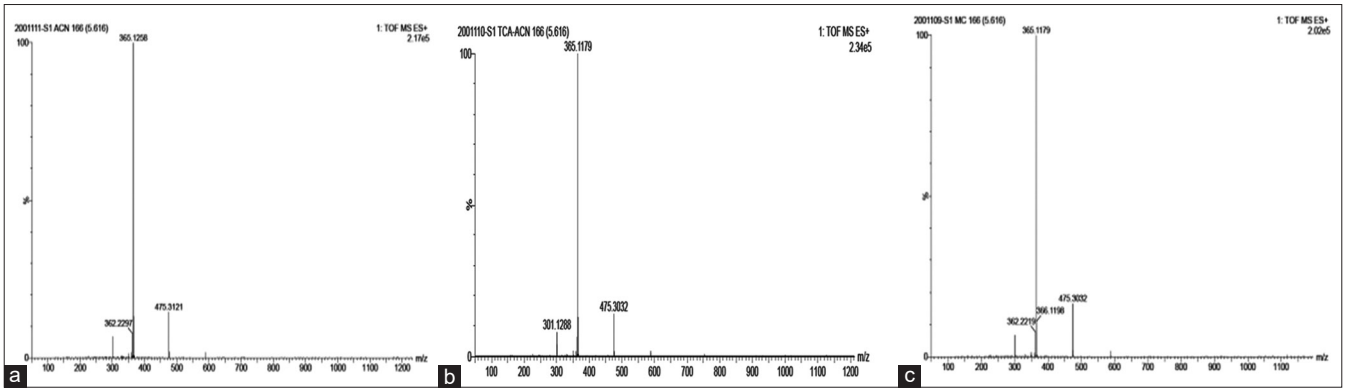

Figure 5: Elution time at 5.6 min of Liquid chromatography – Mass spectrophotometer (a) acetone precipitation, (b) trichloro acetic acid, and (c) methanol/chloroform precipitation methods, respectively

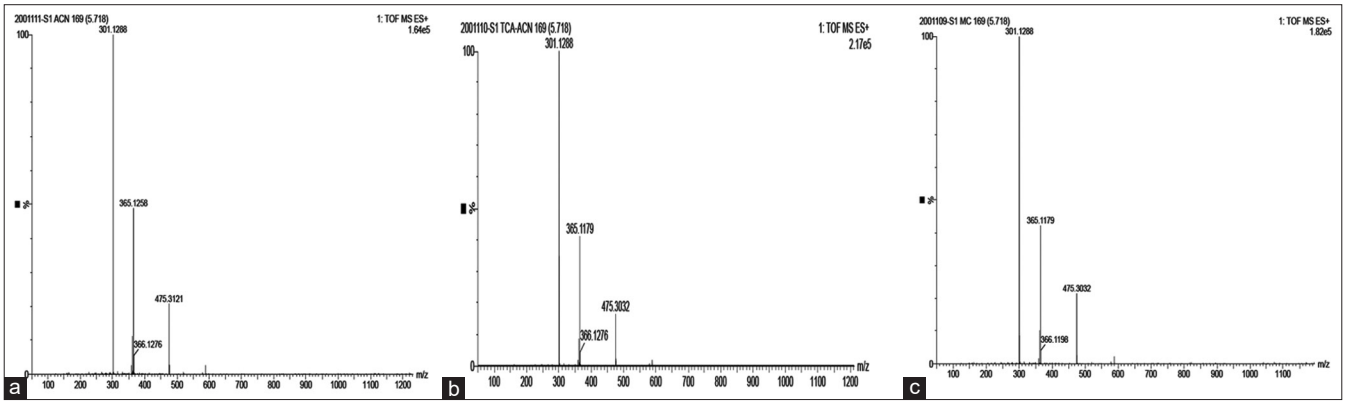

Figure 6: Elution time at 5.7 min of Liquid chromatography – Mass spectrophotometer (a) acetone precipitation, (b) trichloro acetic acid, and (c) methanol/chloroform precipitation methods, respectively

The LC-MS results of Sample 2 (S2) showed that Figure 9a-c 104Da, 122Da, and 144Da are similar and 277Da and 307Da are unique at 0.7 min. Figure 10a-c showed that 104Da and 122Da are similar in all three methods at 0.8 min. Figure 11a-c showed that 362Da, 475Da, and 476Da are similar and 142Da and 301Da are unique at 3.4 min. Figure 12a-c showed that 301Da, 365Da, 366Da, and 475Da are similar and 350Da and 362Da are unique at 5.6 min. Figure 13a-c showed that 301Da and 425Da are similar and 426Da, 827Da, 315Da, 365Da, 475Da, and 476Da are unique at 5.9 min.

We looked at the proteins that were common and distinctive distinctive in the samples of each method [Figure 14]. About 17.6% of proteins were shared among the three approaches in terms of percentage of proteins detected. A comparison of two alternative methodologies is made between acetone and methanol/chloroform precipitation, indicated the highest amount of protein that was shared as 69%, TCA/acetone and methanol/chloroform showed 63.8% and acetone and TCA/acetone precipitation showed 52.7%. M/C, TCA/acetone, and acetone precipitations showed 26%, 11.7%,

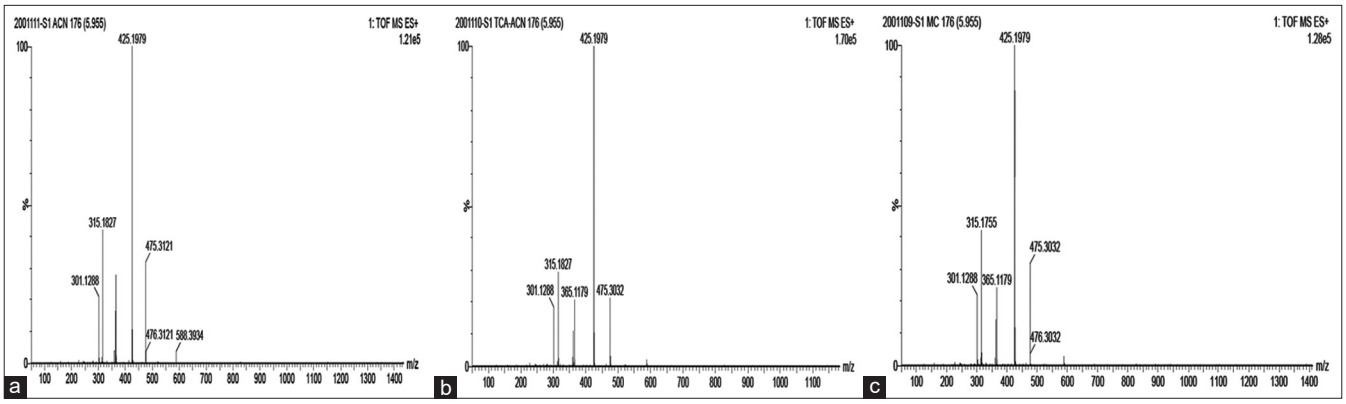

Figure 7: Elution time at 5.9 min of Liquid chromatography – Mass spectrophotometer (a) acetone precipitation, (b) trichloro-acetic acid, and (c) methanol/chloroform precipitation methods, respectively

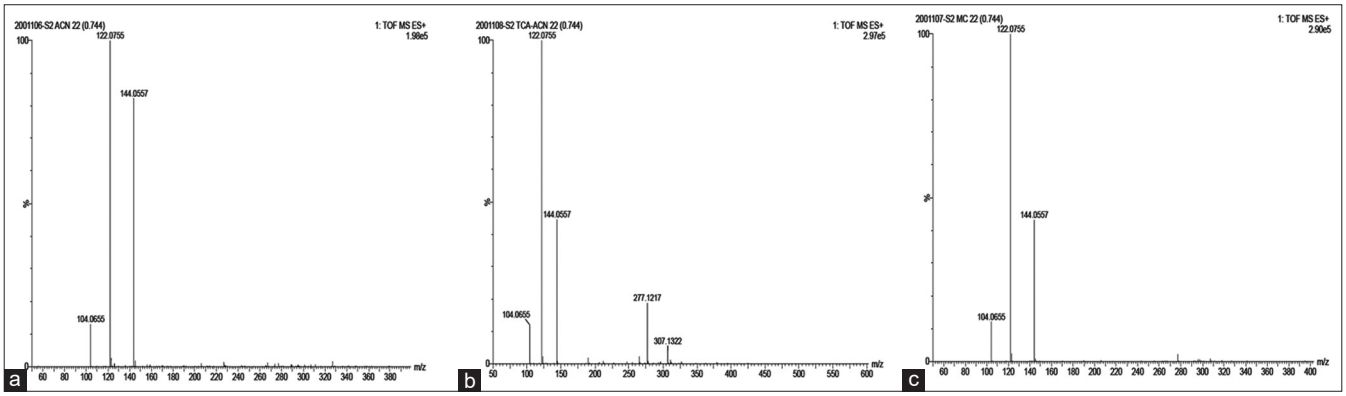

Figure 8: Elution time at 0.7 min of Liquid chromatography – Mass spectrophotometer (a) acetone precipitation, (b) trichloro acetic acid, and (c) methanol/chloroform precipitation methods, respectively

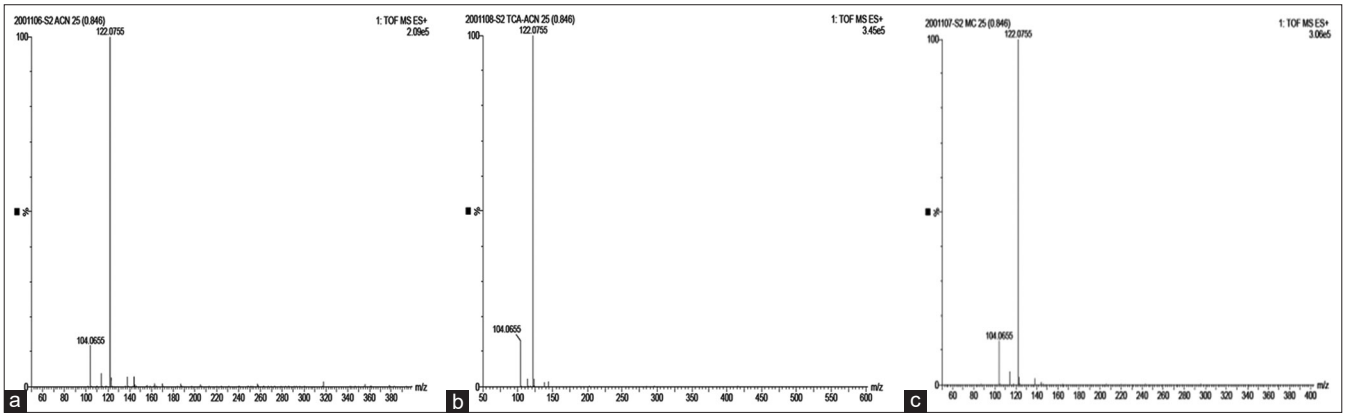

Figure 9: Elution time at 0.8 min of Liquid chromatography – Mass spectrophotometer (a) acetone precipitation, (b) trichloro acetic acid, and (c) methanol/chloroform precipitation methods, respectively

and 12.4% of the unique proteins excluding shared proteins, respectively. Among the three methods M/C method showed the uppermost value.

As a result, urinary protein produced by methanol/chloroform precipitation offers the finest chance of yielding an outstanding outcome in the identification of a protein using LC-MS/MS-based analysis of urinary proteins.

Protein recovery check

In triplicate, the amounts of protein in the samples were measured using Lowry's method of protein estimation and were resulted as 280 µg/ml and 290 µg/ml. Then samples were processed for three different precipitation methods. In terms of protein content before and following precipitation, the efficacy of precipitations was solid. The reported results

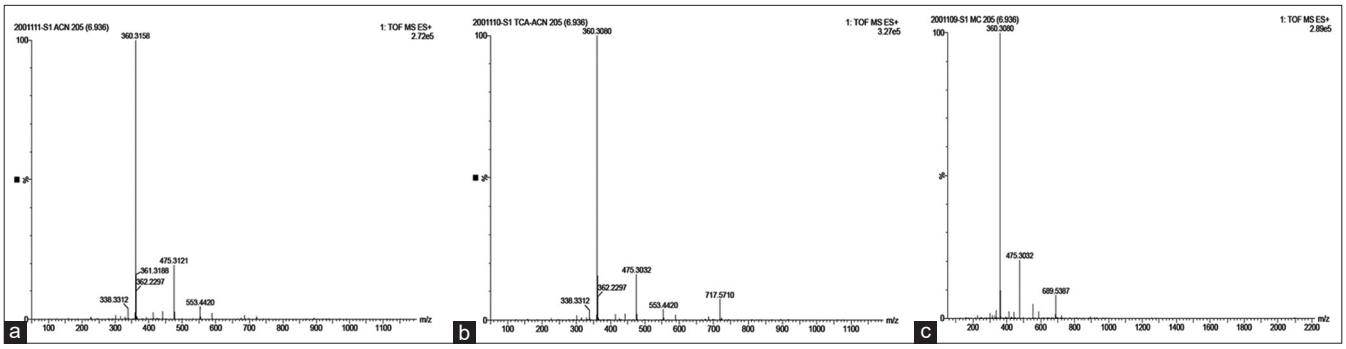

Figure 10: Elution time at 6.9 min of Liquid chromatography – Mass spectrophotometer (a) acetone precipitation, (b) trichloro acetic acid, and (c) methanol/chloroform precipitation methods, respectively.

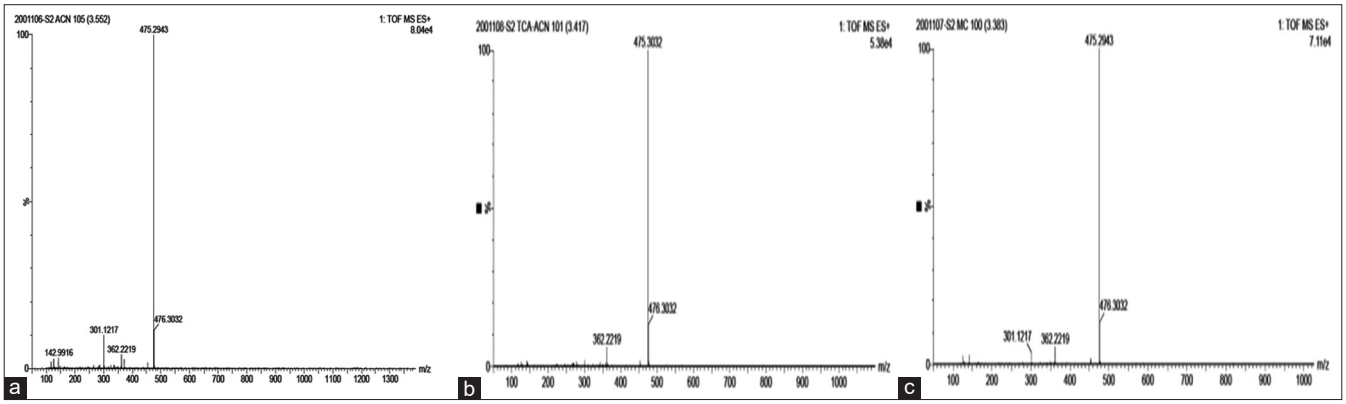

Figure 11: Elution time at 3.4 min of liquid chromatography – mass spectrophotometer (a) acetone precipitation, (b) trichloro acetic acid, and (c) methanol/chloroform precipitation methods, respectively

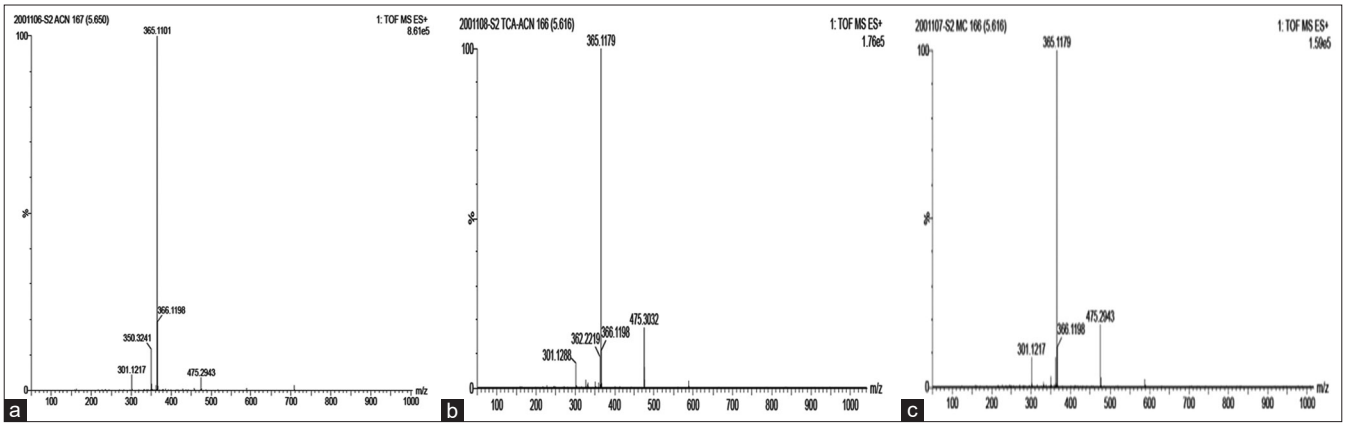

Figure 12: Elution time at 5.6 min of liquid chromatography – mass spectrophotometer (a) acetone precipitation, (b) trichloro acetic acid, and (c) methanol/chloroform precipitation methods, respectively

are a composite of at least three experiments. Following the supernatant examination, the Lowry's test was employed to evaluate the protein concentration. The following formula is used to calculate the protein recovery from Precipitated urine.

$$\text{Protein recovery rate R}(\%) = \frac{\text{Pppt(precioitated protein amount)}}{\text{Pu(Urine protein amount)}} \times 100 \quad (1)$$

The samples showed good findings in LC-MS/MS and had a high protein recovery rate (about 80% or greater). According to the findings of this investigation, three methods showed a high recovery rate in the sample precipitation [Table 1]. The highest value (90%) was found in M/C precipitation, with a maximum value of above 90%. On an average, acetone, and TCA/acetone precipitation recovered 68.2% and 74.8% of urine protein, respectively. In each precipitation method, the size of protein pellets precipitated from urine was consistent with the rate with

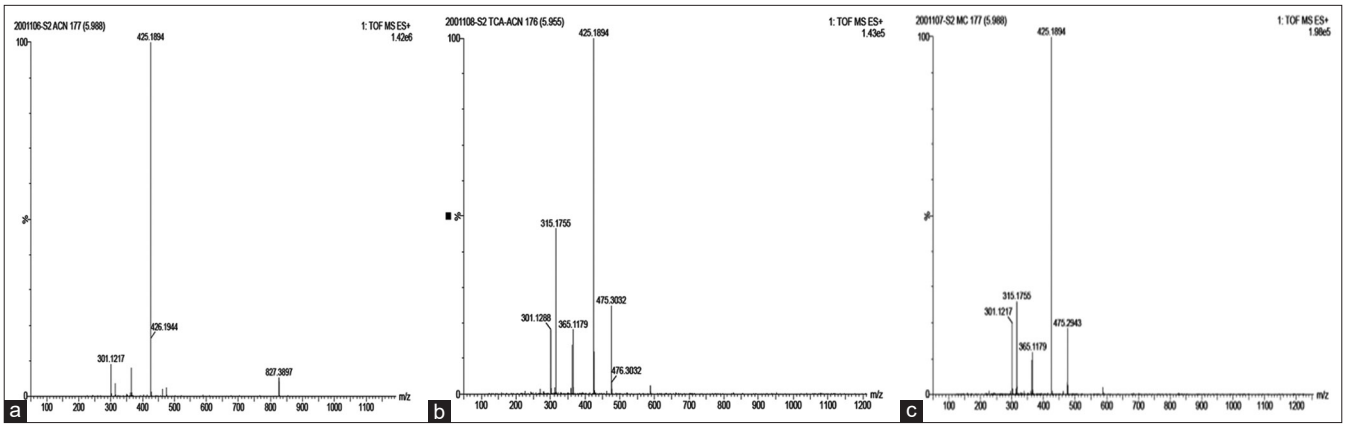

Figure 13: Elution time at 5.9 min of liquid chromatography – mass spectrophotometer (a) acetone precipitation, (b) trichloro acetic acid, and (c) methanol/chloroform precipitation methods, respectively

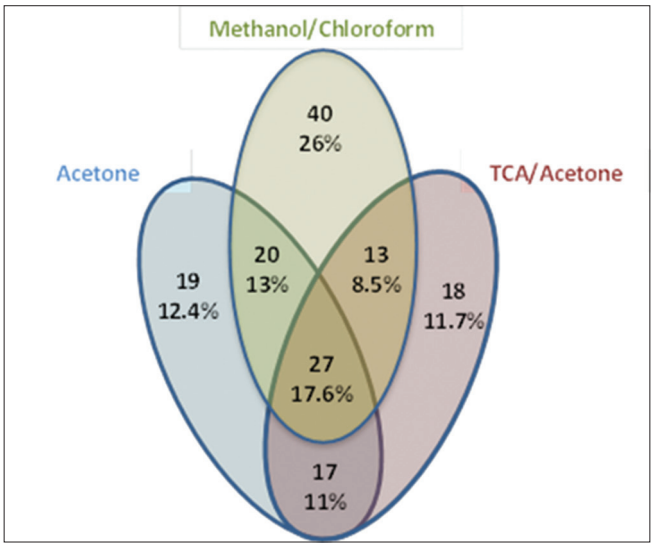

Figure 14: The shared and unique protein in each precipitation method. Independent proteomic data provided from each precipitation were analyzed for the isolation of common or unique protein in each group. These data were analyzed by Venn diagram to indicate shred protein and unique protein between four different precipitation methods

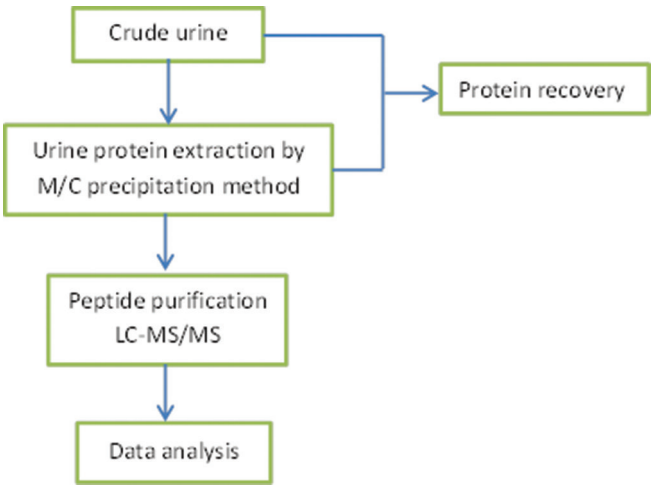

Figure 15: An optimized workflow of urinary protein preparation using liquid chromatography – mass spectrophotometer (MS)/MS analysis

Table 1: Percentage of protein recovery

Precipitation method	Protein amount before precipitation (mg)	Protein amount after precipitation (mg)	Percentage of recovery (%)
Chloroform/ methanol	280.11±30.42	252.66±53.86	90.20±12.31
Acetone	280.11±30.42	190.88±45.63	68.14±11.13
10%TCA/ acetone	280.11±30.42	209.45±19.54	74.77±19.65

the recovery value rate. Throughout the study, a LC-MS/MS-based urine proteomics sample preparation workflow has been established [Figure 15].

Low solubility of urine proteins precipitated was another issue, because the proteins recovered from urine by precipitation using organic solvents were commonly desiccated, the pellet proved difficult to dissolve in 8 M urea/50 mM Tris-HCl (pH 8.0), a common proteomics buffer.[14] In this state, a hazy white coating at the bottom of the tube remained, which was thought to contain undissolved proteins. The 8 M urea/50 mM Tris-HCl, 50 mM EDTA (pH 8.0) buffer was used to solve this difficulty in urine protein production. When compared to conventional buffers without EDTA, this buffer helped the protein dissolve completely. Improvements in protein dissolving resulted in a considerable improvement in the performance of LC-MS/MS analysis, resulting in a considerable increase in the identified protein number.

DISCUSSION

The efficiency of the protein precipitation methods was studied by comparing three precipitation methods to evaluate a greater number of proteins present in healthy human urine samples using organic solvents such as acetone, TCA/acetone, and chloroform/methanol. We chose the method since it has been

widely regarded as one of the standard approaches over a long period of time. LC-MS/MS has become the method of choice for detecting the protein contents of complicated biological materials with excellent accuracy and sensitivity. It is also a crucial step in identifying proteins in samples, not just dominant ones yet there are also smaller ones. The precipitated urine samples of each method were analyzed by LC-MS/MS and the results were obtained with a good number of retention peaks. The results achieved using the chloroform/methanol mixtures were superior to those obtained using acetone and TCA/acetone among the three techniques of precipitation tested. Acetone precipitation has the advantage of being a practical practical process, but it does require a considerable volume of organic solvent. When compared to acetone precipitation, TCA/acetone and chloroform/methanol precipitation resulted in approximately a two-fold lesser recovery.

A recent study compared precipitation methodologies,[14,16] which were carried out using healthy human urine, it was revealed that ethanol, acetone, TCA/acetone, and chloroform/methanol precipitations resulted a greater protein recoveries compared to other precipitation methods. Protein extraction from bio-fluid samples, such as urine, is typically accomplished using M/C precipitation. We underline that the mixture of Tris-HCl pre-treatment and M/C precipitation is the most efficient method for extracting protein from urine to overcome the low solubility of precipitated urine proteins.

Our goal in this study was to find the best approach for getting the most yields from urine samples. To do so, three distinct strategies were thoroughly explored to find the optimum protein precipitation method. We found that methanol/chloroform was one of the best protocols. However, because the precipitation procedures are very dependent on the starting material, acetone, and TCA/acetone also performed well.

CONCLUSION

This research helps to develop a standard approach for protein study in urine samples. It has a broad range of applications employed in urine proteomics, not only for diagnosis, but also for basic biological research in fields such as physiology and pharmacology, using urine biomarkers. In addition, when the procedure is used by each study in future, it adds to the creation of a urine proteome database.

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