Surface-Enhanced Laser Desorption/Ionization-Time of Flight-Mass Spectrometry (SELDI-TOF-MS): A New Proteomic Urinary Test for Patients With Urolithiasis

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SELDI-TOF-MS is a highly sensitive protein-analysis tool capable of detecting minute protein profile differences between biological samples. As proteins have been associated with urinary tract calculi, protein-based urinalysis may offer insights into their diagnosis. The purpose of this study was to evaluate SELDI-TOF-MS as a potential method for identifying urinary biomarkers of urolithiasis.

Midstream sterile urine samples were obtained from 25 male patients with a confirmed diagnosis of urolithiasis (test group) and 25 male subjects with no known history of the disease (controls). Urinary levels of oxalate, total protein, albumin, and osteopontin were determined. Protein profiles were generated using SELDI-TOF-MS.

Key words: urinary tract calculi; albumin; protein profiling; diagnostics; urine

SELDI-TOF-MS profiling revealed a relationship between protein peak intensities at 67 and 24 kDa that differed between the two groups. The ratio of p67:p24 was found to be less than 1.0 in all of the control samples (mean 0.26), while 18 out of 25 (72%) of the test group samples displayed a ratio greater than 1.0 (total group mean 4.75, $P < 0.001$). Albumin, total protein, and oxalate levels were higher in the test group than the controls.

Although SELDI-TOF-MS is not yet in widespread use in hospital and diagnostic laboratories, this system represents a promising new method for rapidly identifying patients with urolithiasis. *J. Clin. Lab. Anal.* 18:170–175, 2004. © 2004 Wiley-Liss, Inc.

INTRODUCTION

Urinary tract calculi are among the most common renal disorders, affecting approximately one in eight people (1). Although the majority of calculi will pass spontaneously, a significant number will require intervention. Treatment options include extracorporeal shock wave lithotripsy (SWL), percutaneous nephrolithotripsy, ureteroscopy, and, rarely, open surgery. While there have been significant improvements in treatment, similar advances in screening have not

occurred. Consequently, there is currently no universally

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accepted rapid urine test in clinical use to identify patients either having, or at risk of developing, renal calculi or renal colic. Methods of diagnosis (and potential screening tests) available today include urinalysis, urine microscopy, urine chemistry screen, serum chemistry screen, and imaging studies such as plain abdominal radiography of kidneys, ureters and bladder (KUB), intravenous pyelography (IVP), retrograde pyelography, ultrasound (U/S), and computed tomography (CT) scan. Unfortunately, these tests are expensive, time consuming, and may be associated with risks such as radiation exposure and contrast reactions [2]. Furthermore, they lack adequate sensitivity, specificity, and a positive predictive value necessary for a good screening test.

Surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS) is a relatively new and highly sensitive proteomic tool used for the study of proteins within biological samples. In this technique, samples are mixed with an energy absorbing molecule (matrix-low molecular weight aromatic compounds that absorb light at the same wavelength as the laser) and air dried on metallic chips possessing various surface characteristics (i.e., hydrophobic, hydrophilic, anionic, cationic). Laser energy is then applied to the sample/matrix crystals. This ionizes the proteins within the sample and results in their desorption from the chip surface. Finally, the proteins are quantitated based upon size via mass spectrometry. Among its attributes, SELDI-TOF-MS can generate extensive protein profiles rapidly and accurately from a minimal amount of sample and the use of surface-enhanced chips can aid in the identification, isolation, and characterization of specific peptides. This tool has proven useful in the identification of biosurfactant proteins from lactobacilli (3,4) and has recently been studied as a potential screening tool for patients with malignancies of the urinary tract (5). The aim of this study was to use SELDI-TOF-MS in an attempt to identify urinary protein profile changes associated with urolithiasis.

MATERIALS AND METHODS

Midstream urine samples were obtained from all subjects. The test group consisted of 25 male patients with a clinical and radiographic diagnosis of urolithiasis that were referred for SWL. Radiographic imaging modalities used to confirm the diagnosis included KUB, IVP, CT scan, or U/S. The control group consisted of 25 male volunteer subjects with no known history of urolithiasis. All samples were filter-sterilized and stored at -80°C until analysis.

Urinary protein and oxalate determination assays were performed at room temperature using 96-well

microtiter plates (VWR, Mississauga, Canada). Total urinary protein was determined using a modified benzethonium chloride assay (6). Briefly, 127.5 μL of benzethonium chloride (Sigma, Oakville, Canada) solution (2 mg/mL), 31.5 μL of ethylenediaminetetra acetic acid (EDTA; Sigma) solution (33 mM in 0.5 mM NaOH; Sigma), and 10 μL of urine were added to each well. They were then mixed and incubated for 5 min. Sample turbidity, as a function of total protein content, was measured at 575 nm using a microplate reader (Biorad, Mississauga, Canada) and the results compared to values obtained using bovine serum albumin standards (BSA) (Sigma). Total urinary oxalate was determined using an Oxalate Determination Kit (Sigma) following the manufacturer's supplied protocol, scaled down such that 10 μL of urine was analyzed. Sample readings were taken at 575 nm and compared to those acquired using ammonium oxalate (Sigma) standards.

For SELDI-TOF-MS analysis, 2 μL of each urine sample was applied to a gold-coated ProteinChip[®] array (Ciphergen Biosystems, Inc., Fremont, CA) along with 2 μL of a saturated solution of 3,5-dimethoxy-4-hydroxycinnamic (sinapinic) acid (in 50% acetonitrile, 0.5% trifluoroacetic acid) (Sigma) and air dried at 37°C for 5–10 min. Samples were analyzed using the SELDI-TOF technique (Protein Biology System II, Ciphergen Biosystems), and protein mass identification was performed by averaging 35 laser shots (laser intensities utilized were 220, 250, 275, and 295) acquired from various areas of the ProteinChip[®] surface. Only those peaks possessing a signal to noise ratio greater than 3.0 were considered to be "true" peaks. Samples that failed to yield readable spectra (generally due to high protein content) were diluted up to 10-fold in sterile water and retested. Additionally, 10 samples from each group were reanalyzed in a separate experiment and compared to the initial results to determine the level of intra-sample variability.

Urinary albumin was quantified via enzyme-linked immunosorbent assay (ELISA) in 96-well microtiter plates as follows. Serial dilutions of each urine sample were prepared in carbonate-bicarbonate buffer (50 mM Na_2CO_3 and 50 mM NaHCO_3 [Sigma], combined until pH = 9.6) such that the total volume in each well was 100 μL . Plates were incubated overnight at 4°C to allow protein binding, samples discarded, and the wells washed three times using excess phosphate-buffered saline (pH 7.3) containing 0.05% Tween 20 (Sigma) (PBS-T). Plates were incubated sequentially with a polyclonal rabbit anti-human serum albumin antiserum (Dako, Mississauga, Canada) (1:2,000 dilution) and a polyclonal goat anti-rabbit IgG antiserum conjugated to horseradish peroxidase (Sigma) (1:10,000 dilution), both in PBS-T containing 2% skim milk

powder (Sigma) (100 μL /well, 1 hr, 37°C). Following each incubation, plates were washed three times using excess PBS-T. In the final step, wells were incubated with 100 μL colorimetric solution (116 $\mu\text{g}/\text{mL}$ 3,3',5,5' tetramethylbenzidine in 50 mM sodium acetate buffer [pH 6.0] containing 0.03% H_2O_2) at room temperature until the desired color development point was reached. The reaction was stopped with 100 μL 12.5% H_2SO_4 (Sigma). Absorbance was read at 450 nm and compared to dilutions of human serum albumin (Sigma). Urinary osteopontin was quantified via ELISA as previously described (7). Two-tailed student's *t*-test was employed to determine the significance ($P < 0.05$) between the test and control groups' observed values.

SDS-PAGE and Western blotting were conducted on 15 urine samples from each group using the polyclonal rabbit anti-HSA antiserum described above. Five micrograms of total protein from each sample were separated via 10% denaturing SDS-PAGE using standard protocols and transferred to sequencing grade PVDF membrane (Amersham Pharmacia Piscataway, NJ) using a Mini Trans-Blot[®] Electrophoretic Transfer Cell (Biorad) as per the manufacturer's instructions. Blots were blocked for 1 hr in PBS-T containing 2% skim milk powder and incubated sequentially in the rabbit anti-HSA and goat anti-rabbit antisera as described above. Following each incubation, blots were rinsed three times (10 min each) in excess PBS-T. Detection was performed using 50 mL of the colorimetric solution described above and color development was arrested by the addition of an equal volume of 12.5% H_2SO_4 . HSA was used as a positive control.

RESULTS

Mean urinary protein (540.5 vs. 170.5 $\mu\text{g}/\text{mL}$, $P = 0.0002$) and oxalate (209.8 vs. 158 μM , $P = 0.0442$) levels were found to be significantly higher among the test group than the controls, respectively (Table 1). This corresponded to 19 out of 25 (76%) stone patients and 4

out of 25 (16%) controls displaying clinical proteinuria ($> 250 \mu\text{g}/\text{mL}$).

Protein profiling via SELDI-TOF-MS revealed several interesting findings. The most important of these was the substantial variation among samples in the relative intensity of a peak located at $\approx 67 \text{ kDa}$ (Fig. 1). When compared to that of a second peak at $\approx 24 \text{ kDa}$ common to all samples (termed p24) and expressed as a ratio, the change in p67 intensity was found to be significantly different between the two groups. The ratio of p67:p24 was less than 1.0 in all of the control samples (mean 0.26), while 18 out of 25 (72%) in the test group displayed a ratio greater than 1.0 (total group mean 4.75) ($P = 0.0001$) (Table 1). Ratios were found to be identical among duplicate samples, denoting little intra-sample variability.

In Fig. 1, A and C represent the single charged ($m + \text{H}^+$) peaks p24 and p67, respectively. Assignment of the peaks to 24 and 67 kDa was based upon the average of all samples from the two groups, not only those shown in the figure. B represents a double charged ($m + 2\text{H}^+$) form of peak p67 that can occur in SELDI-TOF-MS profiling when high levels of a protein are present. Peaks at ≈ 39 and $\approx 49\text{--}52 \text{ kDa}$ observed among the control group samples were also found in those of the test group. However, they are obscured in the figure due to the high relative intensity of p67 among those samples.

Since both albumin and osteopontin fall within the size range of p67, we quantified both proteins via ELISA to determine if either of their levels differed significantly between the two groups. Although osteopontin levels were found to be similar between the test group and controls, a finding consistent with previously published data (7), albumin levels showed a marked increase among those with urolithiasis (Table 1). While the average albumin content in the control group was 4.74 $\mu\text{g}/\text{mL}$, accounting for 5.04% of the total protein present, these values were significantly higher among the stone patients at 134.45 $\mu\text{g}/\text{mL}$ ($P = 0.0146$) and 16.16% ($P = 0.0041$), respectively (Table 1).

Table 1. Urinary data comparisons between the urolithiasis and control groups

	Urolithiasis group	Control group	P value
Mean protein, $\mu\text{g}/\text{mL}$ (range)	540.5 (75–1830)	170.5 (33–760)	0.0002
No. of subjects with proteinuria ^a	19/25	4/25	NA
Mean oxalate, μM (range)	209.8 (68–409)	158.0 (48–377)	0.0442
Mean p67:p24 ratio (range)	4.75 (0.08–22.0)	0.26 (0.07–0.80)	0.0001
No. of subjects with p67:p24 ratio greater than 1.0	18/25	0/25	NA
Mean albumin, $\mu\text{g}/\text{mL}$ (range)	134.45 (0.5–1170)	4.74 (0.2–24.1)	0.0146
Mean % albumin of total protein (range)	16.16 (0.3–63.9)	5.04 (0.1–33)	0.0041
Mean osteopontin, $\mu\text{g}/\text{mL}$ (range)	14.92 (0.5–38.5)	11.94 (1.0–54.6)	0.4145

NA, not applicable.

^aProteinuria refers to $> 250 \mu\text{g}/\text{mL}$ protein content.

Fig. 1. SELDI-TOF-MS protein spectra (size range shown ≈ 20 – 100 kDa) of five representative samples from each of the test and control groups.

Further analysis of the 18 urolithiasis patients with a p67:p24 ratio greater than 1.0 revealed higher levels of both total protein and albumin than those observed when comparing all 25. Of these 18, 16 (89%) demonstrated clinical proteinuria, with an average of $640.67 \mu\text{g/mL}$. Mean albumin content rose to $182.0 \mu\text{g/mL}$, accounting for an average of 20.5% of total protein. Additionally, 13 (72%) of these patients displayed SELDI-TOF-MS peaks at ≈ 130 – 135 kDa and/or ≈ 145 – 150 kDa, peaks not present in any other samples (data not shown). While the former is likely albumin dimers ($2m + H^+$), the identity of the latter is unknown.

To confirm the 67 kDa peak as albumin, SDS-PAGE and Western blotting were performed on 15 urine samples from each group (data not shown) using the polyclonal rabbit anti-HSA antiserum previously employed for the ELISA quantification. As expected, a visible band was observed at 60–65 kDa for all of the samples displaying the 67 kDa SELDI-TOF-MS peak as well as the HSA positive control lane. Although densitometry was not carried out for band intensity quantification, both the presence and visual intensity of the band correlated well with the SELDI-TOF-MS data upon comparison. Notably, two additional bands at ≈ 45 and ≈ 50 kDa were observed in two of the sample lanes and are most likely albumin degradation products.

DISCUSSION

The data demonstrate the potential for proteomic methods to identify markers for urolithiasis. While protein markers are not new to urology (8), one difference here is that in-depth profiles can be analyzed rather than screening for an isolated protein marker. The SELDI-TOF-MS system is relatively inexpensive when compared with other diagnostic tools used in hospital or clinic settings (approximately \$150,000 US). Speed of use is a major advantage as urine collected from patients can be readily processed within 3 hr. Additionally, the sensitivity of the system allows for the analysis of minute volumes and samples with extremely low protein content (ng/mL). Overall, as long as an experienced person reviews the data, results can be obtained within half a day.

The use of peak ratio comparisons in the analysis of samples via mass spectrometry is not novel (9,10) and possesses several advantages. First, the use of ratios ensures that all of the factors and conditions affecting sample peak intensities (such as sample ionization and desorption levels, matrix/sample crystallization) between experiments are similar. Second, they eliminate the need for protein quantification prior to sample analysis, thus reducing time, expense, and potential errors. Finally, they allow useful information to be gathered from one patient sample. Many of the clinical

parameters assessed currently involve time-consuming, 24-hr collections due to overall daily changes in urinary composition, including protein levels. The use of ratios to express protein changes relative to an internal standard (such as p24) can abolish this requirement, again decreasing time and expense.

The selection of p24 as an internal standard was based upon two findings: 1) its ubiquitous presence among all samples, and 2) differences in its relationship to p67 between the test and control groups. Although it is unknown whether p24 is comprised of a single peptide or several proteins of similar size, our unsuccessful attempt to identify it via SDS-PAGE separation and N-terminal sequencing (data not shown) suggests the latter. Future experiments will attempt to identify p24, as well as other peptides, that could collectively serve as a stronger internal standard upon which to base comparisons.

In this study, all samples were applied to a gold chip to generate more "complete" protein profiles, enabling us to look for general differences among the entire sample. In the future, samples will also be applied to surface-enhanced chips for the specific analysis of certain proteins or protein groups. In this way, markers such as that described here can be observed among less background. Furthermore, additional markers may exist, but were not identified here using the gold chip due to obscuring spectra from other peptides of a similar size or higher concentration.

The test had a sensitivity of 72%, specificity of 100%, positive predictive value of 100%, negative predictive value of 78%, and false negative and false positive rates of 28 and 0%, respectively. Although these values suggest that SELDI-TOF-MS has utility in the diagnosis of urolithiasis, further studies are necessary to determine whether the results observed here are specific for the presence or formation of urinary tract calculi. Considering the absence of additional patient groups in this study, it is impossible to discount other potential reasons (urinary tract infections or disorders, hypertension, cancer, diabetes, stent presence) for the observed rise in albumin. Additionally, should future studies support our hypothesis relating the observed SELDI-TOF-MS peak changes to urolithiasis, further work monitoring non-, first time, and recurrent stone-formers, with and without active stones, is required over time to determine its true value as a general screening test for spotting asymptomatic patients in need of further testing. These studies will also determine whether the rise in albumin observed in this study occurred prior to stone formation or after the onset of a clinical stone episode.

Previous studies have commonly observed albumin as a major constituent of urinary tract calculi (11,12). In

addition to our results demonstrating its frequent increase within the urine of stone patients (as well as albumin's ability to self-aggregate, bind numerous urinary proteins [11,13], and attach to calcium oxalate [11,12,14] and calcium phosphate crystals in vitro [12]), this finding suggests that it may play a role in stone formation. However, additional work aimed specifically at albumin's role in the disease is also required. Although screening tests specific for urinary albumin are already in use and are constantly improving in both sensitivity and rapidity, they can give false positives and negatives, especially in patients with very high and low urinary protein levels (15). False positive results can also be caused by numerous proteins other than albumin (16). One of the advantages of using SELDI-TOF-MS peak ratio comparisons is their independence from sample protein levels because the quantity of albumin present is compared directly to other protein(s) within the same sample and expressed as a relative value.

In conclusion, this study suggests that a proteomic SELDI-TOF-MS system has the potential to play an important role in reducing health care and patient costs. It also saves time for clinicians and improves their ability to identify and manage patients with urolithiasis.

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