Capillary electrophoresis-mass spectrometry for targeted and untargeted analysis of the sub-5 kDa urine metabolome of patients with prostate or bladder cancer: A feasibility study

Matthew S. MacLennan, Miranda G.M. Kok, Laiel Soliman, Alan So, Antonio Hurtado-Collar, David D.Y. Chen

ABSTRACT

Targeted and untargeted analyses of the sub-5 kDa urine metabolome of genitourinary cancer patients (prostate and/or bladder) were performed without chemical derivatization using capillary electrophoresis-electrospray ionization-mass spectrometry (CE-ESI-MS). For targeted analysis, endogenous levels of sarcosine and 5 other amino acid metabolites involved in the progression of prostate cancer were quantified in four patients and in a pooled urine sample from healthy volunteers. An untargeted analysis (m/z 50 to 850) of patient urine was performed using the same CE-ESI-MS system identifying over 400 distinct molecular features per patient. All patient urine samples were collected at prostatectomy/cystectomy via catheter. Patient urine samples were filtered by centrifugation, with endogenous sarcosine enriched by solid-phase extraction, and the processed samples loaded onto CE-ESI-MS for analysis. Diagnostic information, digital pathological slides, and tissue samples were collected and stored in a comprehensive biobanking database. The introduction of urine sample collection into the surgery workflow was facile and is a promising strategy for addressing the translational research challenge of moving smoothly from “chromatogram to nomogram”.

Keywords:
- Capillary electrophoresis
- Prostate cancer
- Urine
- Metabolomics
- Bladder cancer
- Amino acids

1. Introduction

With the advent of metabolomic profiling, the search for relevant biomarkers for a variety of diseases has intensified. In particular, the effectiveness of using metabolomic biomarkers as screening tools in various fields of oncology is being continuously investigated [1–3]. In 1987, the measurement of prostate-specific antigen (PSA) was introduced as a diagnostic biomarker for prostate cancer by Stamey et al. [4] Although there have been mixed reports on the diagnostic effectiveness of PSA, the general consensus is that PSA is useful for the diagnosis of prostate cancer and it has become a mainstay for screening [5,6]. Although a variety of biofluids have been considered, urine remains an attractive source of potential biomarkers because of its abundance and non-invasive sampling.

For the past decade, sarcosine (N-methylglycine) has been the target of multiple investigations concerning its relationship to the presence and progression of prostate cancer (PCa). The first comprehensive report of the correlation between increased urine concentration of sarcosine and PCa progression was made by Sreekumar et al. [7] In their study, over 1000 metabolites were profiled on prostate tissue, urine and plasma samples, using LC-MS and GC–MS. Since then, a stronger push has been made to incorporate sarcosine into a multiplex marker in urine for detecting the presence and progress of PCa [7,8].

In contrast, an epidemiological study from Norway published in 2014 indicated that higher serum concentrations of sarcosine and glycine were associated with reduced prostate cancer risk in a patient pool of 6000 individuals [9]. The authors are aware of these opposing findings and the role of sarcosine in cancer diagnostics still need to be further investigated. For many other cancers, such as bladder cancer (BCa), no single biomarker has maintained significant diagnostic status, and studies have implicated a wide range of metabolites [3,10]. In 2012, Soliman et al. published a validated capillary electrophoresis-electrospray-tandem mass spectrometry (CE-ESI-MS/MS) method used for quantifying endogenous levels of six underivatized amino acid metabolites, including sarcosine, in pooled healthy urine samples, employing only a single centrifugation step [11]. Applying the same methodology to individual urine samples from subjects with
pathologically confirmed cancers poses a different analytical challenge, from sample collection to analysis to diagnosis. In the presented study, underivatized urine metabolites are separated by capillary electrophoresis and detected by mass spectrometry.

1.1. Urine sampling in the surgical workflow

The Vancouver Prostate Centre implements a Genitourinary Biobanking Management System for storing tissue samples from prostatectomy and integrates digital histopathology with epidemiological information. The urine samples in this study were collected from patients undergoing prostatectomy or cystectomy. Fig. 1 shows a schematic for the integration of pathological and diagnostic data in the Genitourinary Biobanking Management System with metabolomics data obtained from this study. The inclusion of digital pathological information into the overall data structure allows for relevant spatial pathological data, such as tumour proximity to ureter (in the case of bladder cancer), to be correlated to urine metabolite data, increasing the diagnostic accuracy of metabolomics.

1.2. Comparison of analytical methodologies

In the two studies most relevant to the present one [7,9], the analytical methodologies used differ from each other and from those presented here, despite all being some type of separation method coupled to mass spectrometry. We place the current methodology alongside for comparison (Table 1).

2. Materials and methods

2.1. Ethical statement

All patients with prostate and/or bladder cancers who were involved in the Vancouver Prostate Centre Genitourinary (GU) Biobanking Project signed an ethically approved consent form for collection and subsequent analysis of urine.

2.2. Urine sample collection

According to Saude and Sykes, the bacterial levels and the number of freeze-thaw cycles constitute two major confounding factors to the chemical integrity of urine, especially in terms of metabolomic studies [12]. Urine samples were self-obtained from 20 healthy volunteers between the ages of 23 to 30 years and were mixed to form the pooled urine sample. The samples were self-collected presumably midstream urination with no extra chemical sterilization of the vessel prior to sample collection. To constitute the pooled urine sample, the 20 healthy volunteer samples were mixed together. The pooled urine sample was divided into 1 mL aliquots and stored at −20 °C on-site (Table 2).

The four cancer patient urine samples were collected at prostatectomy or cystectomy by urologists at Vancouver General Hospital, obtained from patients possessing pathologically confirmed carcinoma of either the bladder or the prostate, after application of surgical skin rub of 2% chlorhexidine in alcohol. Urine was divided into 1.5 mL aliquots and stored at −80 °C. The urine actively used in analysis was delivered into 2 mL vials and stored at −20 °C for practical purposes.

2.3. Pathological analysis of cancer tissues

The pathological analysis of neoplasms constitutes a type of spatial information. This fact has been taken advantage of with the recent rise in applications of imaging technology to histopathological samples and diagnosis [13-17]. Histopathological evaluation is generally divided into staging and grading, where staging refers to an assessment of the development of cancer spreading throughout the body and grading refers to the cells’ appearance in a tumour, or other carcinoma-specific qualities.
The localized bladder cancers were excised from the bladder and sectioned. Entire prostate glands were sectioned. Tissue sections were stained with hematoxylin and eosin (H + E) and analyzed under light microscopy. Digital images of the paraffin mounts were stored in the Distiller SlidePath data management system (Leica Biosystems). Patient A had superficial bladder cancer (transitional cell carcinoma, or TCC), the most common form of bladder cancer in Americans, which follows three general stages [18]. It is also graded pathologically from I (least developed) to III (fully developed). Squamous cell carcinoma of the bladder, exhibited in Patient B accounts for about 5% of American cases and represents cancer present in the squamous epithelium of the bladder [18]. For Patients C, D, and E, the Gleason score was applied to the stained prostate tissue used to estimate the grade of prostate cancer [19]. Patient C was not studied here due to the presence of blood in the urine sample. Table 3 shows the diagnoses of the patients in this study.

### 2.4. Patient diagnoses

Patient diagnoses at prostatectomy/cystectomy as pertaining to this study are given in Table 3. Patient C was not analyzed due to the presence of blood in the urine.

### 2.5. Chemicals and reagents

All metabolite standards with minimum of 98% purity were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Internal standard sarcosine-D3·hydrochloride was obtained from Toronto Research Chemicals Inc. (North York, ON, Canada) and Sigma Chemical Co., respectively. Formic acid (88%), NaOH, HCl, methanol (HPLC grade),
and glacial acetic acid were purchased from Fisher Scientific (Nepean, ON, Canada). Reagent grade picric acid (98%, 35% water) for creatinine analysis was also obtained from Sigma Chemical Co. (St. Louis, MO, USA). Polymethoxylenimine (PEI) capillary coating reagent of trimethoxysilylpropyl-modified polyethyleneimine, 50% in isopropanol were purchased from Gelest Inc. (Morrisville, PA, USA).

2.6. Preparation of standard stock solutions

Leucine and kynurenine were dissolved in varying volumes of 0.1 M HCl and diluted to volume with water. Because the analysis of sarcosine is separate from the analysis of the other amino acids, there were two standard solutions series: The series of standard solutions for

![Fig. 2. Set of extracted ion chromatograms (XIC) from Patient B obtained by CE-ESI-MS/MS, multiple reaction monitoring (MRM) mode. Notice the relative intensities of sarcosine and alanine and the magnified inset.](image)

Table 4

Endogenous concentrations of the various metabolites in patient urine derived from the x-intercept of a three-point standard addition method with duplicates, using multiple reaction monitoring (MRM) CE-ESI-MS/MS. The standard deviation of the x-intercept is calculated thus: [21]

$$s_{int} = \frac{2}{m} \left( \sum_{i=1}^{m} x_i \right)^{\frac{1}{2}} + \frac{m}{\sum_{i=1}^{m} x_i} \left( \sum_{i=1}^{m} y_i \right)^{\frac{1}{2}}$$

For this experiment, $k = 2$. RSD is calculated as $s_{int}/x$-int.

<table>
<thead>
<tr>
<th>Endo (μmol/L)</th>
<th>Sarc</th>
<th>RSD</th>
<th>L-Pro</th>
<th>RSD</th>
<th>L,L-CysCys</th>
<th>RSD</th>
<th>L-Leu</th>
<th>RSD</th>
<th>L-Glu</th>
<th>RSD</th>
<th>L-Kyn</th>
<th>RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>13.18</td>
<td>9%</td>
<td>7.69</td>
<td>16%</td>
<td>132.8</td>
<td>23%</td>
<td>73.01</td>
<td>15%</td>
<td>11.17</td>
<td>28%</td>
<td>0.11</td>
<td>105%</td>
</tr>
<tr>
<td>B</td>
<td>5.21</td>
<td>21%</td>
<td>4.50</td>
<td>25%</td>
<td>118.1</td>
<td>46%</td>
<td>16.92</td>
<td>41%</td>
<td>21.71</td>
<td>19%</td>
<td>0.11</td>
<td>252%</td>
</tr>
<tr>
<td>D</td>
<td>9.81</td>
<td>14%</td>
<td>2.94</td>
<td>18%</td>
<td>268.2</td>
<td>15%</td>
<td>17.93</td>
<td>19%</td>
<td>11.94</td>
<td>28%</td>
<td>0.092</td>
<td>413%</td>
</tr>
<tr>
<td>E</td>
<td>36.21</td>
<td>20%</td>
<td>11.34</td>
<td>19%</td>
<td>648.0</td>
<td>76%</td>
<td>58.89</td>
<td>10%</td>
<td>42.66</td>
<td>21%</td>
<td>1.65</td>
<td>31%</td>
</tr>
<tr>
<td>POOLED Healthy</td>
<td>0.81</td>
<td>5.99</td>
<td>57.67</td>
<td>17.74</td>
<td>21.55</td>
<td>1.78</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.6. Preparation of standard stock solutions

Leucine and kynurenine were dissolved in varying volumes of 0.1 M HCl and diluted to volume with water. Because the analysis of sarcosine is separate from the analysis of the other amino acids, there were two standard solutions series: The series of standard solutions for
sarcosine—containing sarcosine, and internal standard d3-sarcosine—and the standard solution mixture containing Pro, Cys, Leu, Glu, Kyn, and internal standard d3-Sarc. The pH of these solutions was about 3.9.

2.7. Calibration by standard addition method

The analysis of sarcosine in patient urine required solid-phase extraction, generation of standard curve, and a three point calibration, as described previously [11]. Calibration standard mixtures of the metabolites were prepared. For the non-sarcosine metabolite analysis, all urine samples were filtered through Amicon® Ultra-15 5 kDa Centrifugal Filter Units (Billerica, MA, USA) in a fixed-angle centrifuge at 4000g for 20 min at around 25 °C. An extra several minutes at 10,000g was applied to decrease retentate volume.

2.8. Measurement of creatinine

A Jaffé reaction-based assay on a 96-well plate (8 × 12) was employed using an alkaline picrate solution of 0.00833 M picric acid in 1.67 M NaOH (excess base) to produce a bright yellow solution. Serial dilutions of creatinine standard were added in triplicate to each patient urine sample. The samples were incubated at room temperature for 30 min and subsequently analyzed on a Beckman Coulter DTX880 multimode reader set at 450 nm in absorption mode.

2.9. Instrumentation

2.9.1. Capillary electrophoresis system and software

All experiments were carried out on a PA 800 Plus capillary electrophoresis system (Beckman Coulter, Brea, CA, USA) connected to an AB SCIEX API 4000 triple quadrupole mass spectrometer (AB SCIEX, Framingham, MA, USA). Nitrogen (UHP) was used as curtain and collision gas. A modified capillary cartridge that could accommodate the unique electrospray setup was used for capillary electrophoresis (Maxwell, 2010) [20]. All data acquisition, system control, and integration were performed with Analyst® 1.4.2 software (AB SCIEX, Framingham, MA, USA).

2.9.2. Electrophoretic procedure

CE separations were carried out on a 50 μm inner diameter (I.D.) × 365 μm outer diameter (O.D.) × 85 cm length (L) fused silica capillary (Polymeric Technologies, Phoenix, AZ, USA) coated with cationic polymer trimethoxysilylpropyl polyethyleneimine-HCl in isopropanol (50% v/v). For targeted CE-ESI-MS/MS, a chemical modifier was introduced through a 75 μm (I.D.) × 365 μm (O.D.) × 80 cm (L) bare fused silica capillary at 266 nl/min flow rate. For untargeted CE-ESI-MS analysis, the chemical modifier was introduced via a bare fused-silica capillary (75 μm internal diameter, total length of 80 cm) at a flow rate of 300 nl/min.

For detection of sarcosine in patient samples, the background electrolyte consisted of 0.5% v/v formic acid, 50% v/v methanol and 49.5% v/v water, and the modifier solution consisted of the same. For detection of non-sarcosine amino acid metabolites, the background electrolyte consisted of 2% v/v formic acid, 50% v/v methanol and 48% v/v water, with a modifying solution of the same composition. The same composition was used for untargeted CE-ESI-MS analyses. The 0.5% v/v formic acid buffer has the capability to fully resolve sarcosine from its alanine isomers [11]. The separation of non-sarcosine amino acid metabolites was performed in a 2% formic acid solution in 50% methanol. The sample was injected at 1 psi for 10 s and a voltage of −30 kV was applied. Between injections, the separation capillary was rinsed at 40 psi with methanol for 5 min, air for 1 min, 0.1 M HCl for 5 min, H2O for 5 min, and buffer for 5 min.

2.9.3. ESI-MS/MS and ESI-MS

Electrospray ionization was achieved using a bevelled needle tip geometry with a flow-through microvial [20]. The multiple-ion monitoring (MRM) experiment was carried out on API 4000 triple quad mass spectrometer (AB SCIEX). The MRM parameters were manually set according to Soliman et al. [11] for targeted analyte quantification.

2.10. Data analysis

2.10.1. Targeted metabolite data analysis

For the targeted metabolite analysis, peak areas, migration times, and peak height were calculated using a manual algorithm in the Analyst® 1.4.2 software. Peak area ratios of metabolite to internal standard d3-sarcosine were used to construct a three-point calibration curve (no dilution, 64 × dilution and 32 × dilution) and the x-intercept was interpreted as the vial concentration. The endogenous metabolite concentration was calculated from this.

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Table 5
The endogenous concentrations of metabolites in patient urine (μmol/L) divided by the measured creatinine (mmol/L).

<table>
<thead>
<tr>
<th>µmol/mmol creatinine</th>
<th>Pro</th>
<th>Cys+Cys</th>
<th>Leu</th>
<th>Glu</th>
<th>Kyn</th>
<th>Sarc</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.07</td>
<td>18.48</td>
<td>10.15</td>
<td>1.55</td>
<td>0.016</td>
<td>1.83</td>
</tr>
<tr>
<td>B</td>
<td>0.416</td>
<td>10.91</td>
<td>1.56</td>
<td>2.01</td>
<td>0.010</td>
<td>0.48</td>
</tr>
<tr>
<td>D</td>
<td>0.621</td>
<td>56.70</td>
<td>3.79</td>
<td>2.52</td>
<td>0.019</td>
<td>2.07</td>
</tr>
<tr>
<td>E</td>
<td>0.522</td>
<td>18.40</td>
<td>1.67</td>
<td>1.21</td>
<td>0.047</td>
<td>1.03</td>
</tr>
<tr>
<td>POOLED Healthy</td>
<td>0.740</td>
<td>7.140</td>
<td>2.190</td>
<td>2.660</td>
<td>0.220</td>
<td>0.100</td>
</tr>
</tbody>
</table>

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Table 6
Urinary compounds that were lower (↓) or higher (↑) in concentration in patient samples as compared to the control samples.

<table>
<thead>
<tr>
<th>Compound (m/z)</th>
<th>Migration time (min)</th>
<th>↓/↑</th>
</tr>
</thead>
<tbody>
<tr>
<td>132</td>
<td>16.25</td>
<td>↓</td>
</tr>
<tr>
<td>144</td>
<td>11.41</td>
<td>↓</td>
</tr>
<tr>
<td>156</td>
<td>19.80</td>
<td>↓</td>
</tr>
<tr>
<td>162</td>
<td>17.76</td>
<td>↓</td>
</tr>
<tr>
<td>180</td>
<td>6.50</td>
<td>↑</td>
</tr>
<tr>
<td>212</td>
<td>6.53</td>
<td>↑</td>
</tr>
<tr>
<td>329</td>
<td>8.58</td>
<td>↑</td>
</tr>
<tr>
<td>346</td>
<td>8.61</td>
<td>↑</td>
</tr>
<tr>
<td>389</td>
<td>8.69</td>
<td>↑</td>
</tr>
</tbody>
</table>

* Intensities for one of the patient samples was similar to the intensities of these compounds in the control urine samples.

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Fig. 3. Plot of first 2 principal components (6 PC’s total). The control pooled urine samples (blue circles) were identical in composition; whereas the diseased urine samples (red squares) are each from patients A, B, D, and E. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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2.10.2. Non-targeted metabolite data analysis

For untargeted analyses, MsXelerator software (MsMetrix, Maarsen, The Netherlands) was used to process the full scan data. The obtained metabolic profiles were aligned using the reference peak warping function with the m/z values 76.3, 90.5, 106.5, 122.6, 144.5, 147.6, 162.7 and 186.4 as reference compounds. The maximum allowed difference in m/z between the different metabolite profiles was 0.2 Da. A peak picking procedure was used to determine compounds present in a reference urine sample with a signal-to-noise ratio of 3 and having a migration time between 5 and 30 min. Peak matching was carried out to determine peak areas and heights of common compounds present in all urine samples. Compounds were considered matching if the migration time difference was maximum 0.2 min. Peak areas were normalized to the sum of all peak areas to correct for the difference in urine volume. A table with all normalized areas for the detected urinary compounds was used for principal component analysis (PCA). The PCA loading plot was used to determine discriminatory compounds responsible for the separation of urine obtained from healthy people and patients with prostate or bladder cancer.

3. Results

3.1. Targeted analysis

Fig. 2 shows, as an example, the set of extracted ion chromatograms (XIC) for Patient B, including the XIC for sarcosine. For each standard addition duplicate run (n = 2), peak area ratios (PAR) of targeted metabolites to internal standard (d4-sarcosine) were calculated, averaged, and plotted against the standard concentration. The absolute value of the x-intercept was concentration existing in the vial, which was used to calculate the patient’s endogenous metabolite concentration, shown in Table 4. Endogenous metabolite concentrations normalized to creatinine are shown in Table 5.

3.2. Untargeted analysis

The untargeted analysis of the urine samples resulted in the detection of 468 compounds. The repeatability of the CE-MS method for untargeted urine analysis was assessed by calculating relative standard deviations (RSDs) for the peak area of seven representative urinary compounds spanning the complete metabolomic profile. RSDs were acceptable with values ranging from 2.4 to 16.9%. Peak areas corresponding to compounds were normalized based on the sum of the areas of all detected peaks within a urine sample. Principal component analysis (PCA) using 6 components was performed on the normalized peak areas, and the score plot is shown in Fig. 3. A clear distinction between the urine samples from the healthy volunteers and from the prostate cancer patients is visible. Alterations in urine concentration of at least nine compounds are responsible for this discrimination.

Four compounds showed a lower concentration, whereas the concentrations of five compounds were higher in the urine samples from the patients (Table 6). However, the normalized intensities of m/z value 180 and 212 for one patient appeared to be similar to the intensities of these compounds in the control samples.

4. Discussion

Untargeted metabolomics using CE-MS analysis and subsequent principal components analysis (PCA) revealed a clear difference between urine from healthy volunteers and urine obtained from patients with prostate or bladder cancer. Nine m/z values were singled out as having a significantly higher or lower intensity signal in patient urine than in urine from healthy volunteers. Accurate masses for these compounds could be obtained with high-resolution mass spectrometry. CE-MS was also capable of measuring the levels of proline, cysteine, glutamic acid, leucine, kynurenine and sarcosine in the urine samples with acceptable linearity for all amino acids except cysteine. The variance in peak width of cysteine in the targeted analyses correlated positively with creatinine concentration, suggesting creatinine and like compounds could be responsible for the unreliability of cysteine concentration as reported in this study. The number of patients in this study was too small to make any correlations between metabolite levels and progression of cancer.

We have demonstrated that urine sample collection can occur in the surgical workflow antecedent to the biopsy of cancerous prostate and bladder tissue in order to perform targeted and untargeted metabolomics studies using capillary electrophoresis-mass spectrometry (CE-MS). Future studies will aim to strengthen the connection between urine metabolomic data and pathological information of prostate and associated cancers.

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References


