RAPID COMMUNICATION

Detection and analysis of urinary peptides by on-line liquid chromatography and mass spectrometry: application to patients with renal Fanconi syndrome

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ABSTRACT

Urinary proteomics has become a topical and potentially valuable field of study in relation to normal and abnormal renal function. Filtered bioactive peptides present in high concentration in the nephron of patients with tubular proteinuria may have downstream effects on renal tubular function. In renal Fanconi syndromes, such as Dent’s disease, peptides implicated in altered tubular function or injury have recently been measured in urine by immunochemical methods. However, the limited availability of antibodies means that only certain peptides can be detected in this way. We have used nanoflow liquid chromatography and tandem mass spectrometry (nanoLC-MS/MS) as a complementary technique to analyse urinary peptides. Urine was desalted by solid-phase extraction (SPE) and its peptides were then separated from neutral and acidic compounds by strong cation-exchange chromatography (SCX), which was also used to fractionate the peptide mixture. Fractions from the SCX step were separated further by reversed-phase LC and analysed on-line by MS/MS. Extraction by SPE showed a good recovery of small peptides. We detected over 100 molecular species in urine samples from three individuals with Dent’s disease. In addition to plasma and known urinary proteins, we identified some novel proteins and potentially bioactive peptides in urine from these patients, which were not present in normal urine. These data show that nanoLC-MS/MS complements existing techniques for the identification of polypeptides in urine. This approach is a potentially powerful tool to discover new markers and/or causative factors in renal disease; in addition, its sensitivity may also make it applicable to the direct ultramicroanalysis of renal tubule fluid.

Key words: Dent’s disease, liquid chromatography, mass spectrometry, peptide analysis, proteomics, renal Fanconi syndrome, urine.

Abbreviations: ESI, electrospray ionization; IGF, insulin-like growth factor; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem MS; nanoLC-MS/MS, nanoflow LC-MS/MS; NCBI, National Center for Biotechnology Information; SCX, strong cation-exchange chromatography; SPE, solid-phase extraction.

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INTRODUCTION

Peptides present in the glomerular filtrate may contribute to the progression of renal disease and dysfunction associated with proteinuria [1,2]. Analysis of urinary polypeptides has generally used immunochemical methods [3–5] for quantitative analysis of specific and known biomolecules. However, the need for antibodies means that only well-characterized species can be analysed by this approach; in contrast, mass spectrometry (MS) allows de novo identification of polypeptides [6–8]. When combined with separation techniques, such as liquid chromatography (LC) [9,10] or two-dimensional gel electrophoresis [11,12], MS is well suited to the analytical profiling of complex biological mixtures.

In the present study, we have described a method for the extraction and subsequent qualitative analysis of urinary polypeptides by LC and tandem MS (MS/MS). This approach was used for the analysis of urine from individuals with Dent’s disease, a form of the renal Fanconi syndrome, in which we identified several potentially bioactive peptides that were not present in normal urine.

MATERIALS AND METHODS

Patients

We studied three patients with Dent’s disease, all with clinical, biochemical and molecular genetic characteristics of this disease and creatinine clearances of between 60 and 101 ml/min. The clinical details of these patients have been described elsewhere [3,13,14]. Control urine was collected from three male subjects with no history of renal disease. The study was approved by the local research ethics committee, and all the subjects gave their informed consent.

Extraction of peptides by solid-phase extraction (SPE)

A volume of urine containing 10 μmol of creatinine was added to an equal volume of 1% trifluoroacetic acid. Peptides were extracted with SPE cartridges (OASIS, reversed-phase cartridges, 80 Å pore size; Waters, Milford, MA, U.S.A.) according to the manufacturer’s instructions. Elution was carried out with 80% methanol/0.1% formic acid. Eluted peptides were dried in a SpeedVac and reconstituted in 5% acetonitrile/0.5% formic acid.

Strong cation-exchange (SCX) chromatography

The desalted sample from the SPE step was applied to a SCX column (Polysulphoethyl A, Poly C from Hichrom, Theale, Berkshire, U.K.) packed in a PEEK tube (0.5 mm × 50 mm) as previously described [15,16] and washed with 30 column volumes of a 25% acetonitrile/0.5% formic acid solution. Elution was carried out in 15 steps with increasing concentrations of ammonium acetate (10 mM–1 M) dissolved in 25% acetonitrile/0.5% formic acid. These fractions were dried to remove volatile ammonium acetate. A solution of 100 ng of trypsin dissolved in 15 μl of 25 mM ammonium bicarbonate was added to each of the dried extracts and after incubation overnight at 37 °C, 5 μl of dithiothreitol was added to a final concentration of 3 mM and incubated for 1 h at 50 °C. Reduced peptides were stored at −20 °C until further analysis was performed.

Nanoflow LC-MS/MS (nanoLC-MS/MS)

The peptide mixtures present in one-fourth of each reduced SCX fraction (2.5 μmol of creatinine) were separated by nanoHPLC (Ultimate; LC Packings, Amsterdam, Holland) equipped with a PepMap column (75 μm × 15 cm; LC Packings) at a flow rate of 200 nl/min. Eluting peptides were analysed by electrospray ionization (ESI)-MS/MS in a quadrupole/orthogonal acceleration time-of-flight (Q-Tof) mass spectrometer (Micromass, Wythenshaw, Manchester, U.K.) using a nanoelectrospray ion source and ESI emitters with a 15 μm tapered end (New Objective, Woburn, MA, U.S.A.). This mass spectrometer has been described in detail elsewhere [17]. Briefly, the Q-Tof mass spectrometer consists of two mass analysers, a quadrupole ion filter and a time-of-flight mass analyser (ToF), connected via a collision cell located between these two analysers. In general, mass spectra of ions produced in the ion source by ESI are recorded using the ToF analyser. For MS/MS, multiply charged ion species can be automatically selected and isolated by the quadrupole ion filter and fragmented in the collision cell. The resultant fragment ions will then be measured by the ToF analyser. All parameters are usually chosen for optimized peptide bond fragmentation leading to fragment ions from which the peptide sequence or sequence tags can be deduced for an unambiguous identification of the gene product. The spray tip potential was maintained between 1.5 and 2.2 kV. Gradient elution was performed from 5% to 45% B (95% to 55% A) in 90 min (B, 80% acetonitrile/0.08% formic acid; A, 0.1% formic acid), followed by a wash with 100% B for 10 min. The data-dependent acquisition capability of the Micromass software (MassLynx™) allowed for the collection of approx. 300 MS/MS spectra per chromatographic run under these conditions.

Database search

Data from uninterpreted mass spectra were submitted to Mascot, a software search engine designed for high-throughput and automated interpretation of mass spectrometric data derived from polypeptides [18]. This...
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RESULTS

The analytical strategy detailed above provided what we considered to be an acceptable recovery of small- and medium-sized peptides (60%), while only trace amounts of protein were recovered, as judged by SDS/PAGE and quantitative HPLC (results not shown). This permitted the selective enrichment of small polypeptides, although low levels of larger components (e.g. full-length albumin) were also present (results not shown). Figure 1 illustrates the complexity of the peptide mixture following trypsin...
Figure 2  Distribution of identified urinary gene products from patients with Dent’s disease
Protein ‘hits’ were grouped by arbitrarily selected categories. In normal urine, 27 gene products were found from a pool of three different samples.

Table 1  Selected specific bioactive peptides from urine of patients with Fanconi Syndrome
These peptides were absent from normal urine. The number of peptides refers to tryptic peptides that generated MS/MS data of sufficient quality to derive sequence information.

<table>
<thead>
<tr>
<th>Name</th>
<th>NCBI GI number</th>
<th>Patient 1</th>
<th></th>
<th>Patient 2</th>
<th></th>
<th>Patient 3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Number of peptides</td>
<td>Coverage (%)</td>
<td>Number of peptides</td>
<td>Coverage (%)</td>
<td>Number of peptides</td>
<td>Coverage (%)</td>
</tr>
<tr>
<td>Chemokine member 15 (leukotactin-1)</td>
<td>4759072</td>
<td>1</td>
<td>10</td>
<td>4</td>
<td>61</td>
<td>3</td>
<td>17</td>
</tr>
<tr>
<td>Chemokine CC-3</td>
<td>4759070</td>
<td>3</td>
<td>50</td>
<td>4</td>
<td>48</td>
<td>2</td>
<td>30</td>
</tr>
<tr>
<td>Guanylin</td>
<td>1474271</td>
<td>1</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>IGF-I</td>
<td>755741</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>36</td>
<td>1</td>
<td>21</td>
</tr>
<tr>
<td>IGF-II</td>
<td>1000058</td>
<td>2</td>
<td>49</td>
<td>4</td>
<td>88</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Kallidin II</td>
<td>229138</td>
<td>1</td>
<td>100</td>
<td>1</td>
<td>100</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Dermcidin</td>
<td>16751921</td>
<td>1</td>
<td>12</td>
<td>1</td>
<td>10</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Diazepam-binding inhibitor</td>
<td>10140853</td>
<td>2</td>
<td>23</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>32</td>
</tr>
<tr>
<td>Gastric inhibitory polypeptide</td>
<td>4758436</td>
<td>1</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>Hepcidin antimicrobial peptide</td>
<td>10863973</td>
<td>1</td>
<td>19</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>19</td>
</tr>
<tr>
<td>Platelet basic protein</td>
<td>4505981</td>
<td>5</td>
<td>27</td>
<td>4</td>
<td>19</td>
<td>2</td>
<td>14</td>
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<tr>
<td>Stromal cell-derived factor</td>
<td>13399638</td>
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<td>1</td>
<td>15</td>
</tr>
<tr>
<td>Pigment epithelium-derived factor</td>
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<td>3</td>
<td>3</td>
<td>11</td>
<td>1</td>
<td>3</td>
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</table>

digestion. Inspection of time-of-flight-MS spectra (i.e. unfragmented tryptic peptide ion signals) collected at any point during the chromatographic run showed that several peptides co-eluted from the reversed-phase chromatographic column at any one time. The Micromass software (MassLynx™) allows for the automated selection of peptides for fragmentation (and therefore primary structure determination) when peptide ions above a certain detection level are recorded. Since ESI normally produces multiply charged peptide ions, parameters were chosen so that only multiply charged ions were selected for sequencing by MS/MS. Figure 1 shows four spectra at four different time points of the nanoLC-MS/MS run (at 2 s/spectra). Twenty-six putative peptides were detected, of which 18 were selected for sequencing by MS/MS. Hence, according to the data presented in
Figure 3  Sequence coverage of IGF-II analysed from patients with Dent’s disease
Upper panel: Sequence of IGF-II (NCBI GI number 1000058). Lower panel: Annotated MS/MS spectra of the underlined peptides in the upper panel. 88% of the polypeptide chain was sequenced.

As expected, the majority of polypeptides identified are also present in serum (Figure 2) [20,21]; indeed, 40% of the identified gene products are known plasma proteins. We also identified numerous peptides having potential biological activity (19% in Figure 2) and some of these are presented in Table 1. Since we employed tryptic digestion, this does not mean that the full-length species were necessarily present in urine. However, in the instances in which we sequenced a large percentage of the polypeptide chain, it is reasonable to conclude that the intact peptide was present in the original sample. As an illustration, Figure 3 shows that sequence coverage of 88% was achieved for the insulin-like growth factor (IGF)-II polypeptide chain, which represents almost complete sequence coverage, considering that tryptic peptides with less than four amino acids are usually not recorded. Bradykinin and kallidin II were detected as full-length species. Although these peptides can be generated by trypsin cleavage of their common precursor...
DISCUSSION

We have described a non-immunochemical method for the analysis of peptides in urine based on multidimensional LC-MS. The precision, reproducibility and accuracy of LC-MS for polypeptide analyses are well documented [10,22–25]. In this study, peptides were separated from inorganic and organic salts by reversed-phase SPE and SCX respectively, prior to LC-MS. The SCX step was necessary because many urinary compounds, such as organic acids and bile salts, have retention times in reversed-phase LC within the range of peptides. SCX was also used to separate polypeptides into fractions, because this mode of chromatography provides a robust method for the separation of peptides [26]. Peptides present in the SCX fractions could then be separated further and identified by reversed-phase nanoLC-MS/MS.

Methods reported for the extraction of polypeptides prior to proteomic or mass spectrometric analysis include solvent extraction [27,28], SPE [29,30], ultrafiltration [31,32], ultracentrifugation [5,12], precipitation [12,33], dialysis [34,35], or a combination of these [36]. The limitation of these methods is that some of them fail to recover low-molecular-mass peptides (e.g. dialysis, precipitation, ultracentrifugation and ultrafiltration), while others, by themselves, are not specific for polypeptides (e.g. solid- and liquid-phase extraction). Surface-enhanced laser desorption ionization (‘SELDI’) MS has been used to detect changes in the excretion of urinary polypeptides upon drug administration [37]. However, this method, although useful for profiling urinary polypeptides, fails to characterize fully the species detected in most cases. Using our procedure, we have unequivocally identified more than 100 polypeptides with a large-molecular-mass range: the smallest peptide detected was bradykinin (1.1 kDa) and larger polypeptides, such as complement components and albumin, were also found.

Immunoreactive insulin and parathyroid hormone were detected in the urine of subjects with Dent’s disease [3], but not detected in the present study. A possible reason for this discrepancy could be the limited dynamic range of reversed-phase LC-MS. In data-dependent acquisition experiments, peptide ions above a certain detection level are selected for subsequent fragmentation so that sequence information can be obtained. When several peptides co-elute, the more intense ions are selected preferentially, inducing a bias towards more prevalent peptides. Furthermore, in the electrospray plume generated in ESI, peptides compete for available protons for ionization [38]. When several peptides co-elute from the reversed-phase column, peptides with greater proton affinity ionize more efficiently. Thus, although insulin and its fragments ionize well by ESI, it is possible that the relative abundance of insulin or parathyroid hormone tryptic fragments in relation to other co-eluting peptides was such that these species did not produce enough ions for mass spectrometric detection. Furthermore, smaller polypeptides produce fewer tryptic fragments than large proteins, again reducing the chance of selection for MS/MS. However, in contrast with immunoaffinity-based methods, the advantage of MS is its largely non-discriminative identification of molecular species. Moreover, mass spectrometric analysis does not depend on any presumption as to what peptides may be present in a given biological fluid or the availability of antibodies. In addition, the problem of antibody cross-reactivity and specificity is also avoided by using mass spectrometric methods.

Several ultrafilterable peptides, such as IGF-I (see Table 1), have now been implicated in progressive renal injury and tubulointerstitial disease [39], and others may also directly affect tubular transport mechanisms, as well as cell growth and differentiation. There is now increasing evidence for luminal receptors for a variety of peptide hormones [40,41], which may form part of a finely balanced ‘intracrine’ regulatory system that would be disturbed by the unregulated delivery of luminal polypeptides. In addition, chemokines, such as leukotactin-1, chemokine CC-3 and platelet basic protein (see Table 1), may prove to be useful urinary markers of renal injury or inflammation. Further studies using this analytical approach are needed to explore these possibilities.

Finally, despite the preferential detection of abundant polypeptides in an automated LC-MS/MS analysis, and the competitive nature of peptide ionization, we have demonstrated the use of nanoLC-MS/MS as a highly sensitive, ‘operator-independent’ method of identifying polypeptides in urine. This technique should be useful in complementing immunochemical [3] and electrophoretic [12] approaches to urinary proteomics. It may also prove suitable for the ultramicroanalysis of peptides in renal tubule fluid along the nephron obtained from micro-puncture experiments.
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