


DOI: 10.1373/clinchem.2003.028308

Size-Selective Extraction of Peptides from Urine for Mass Spectrometric Analysis, Glen L. Hortin,‡ Bonnie Meilingere, and Steven K. Drake‡ (Departments of 1 Laboratory Medicine and 2 Critical Care Medicine, Warren Magnuson Clinical Center, NIH, Bethesda, MD; * address correspondence to this author at: Department of Laboratory Medicine, NIH, Bldg 10, Room 2C-407, Bethesda, MD 20892-1508; fax 301-402-1885, e-mail ghorton@mail.cc.nih.gov)

Protein excretion in urine has been suggested as an indicator of kidney disease since the time of Hippocrates. In the early 1800s, Bright further established approaches for studying proteinuria as a marker for kidney disease (1). As methods for quantitative and qualitative analysis have become more sophisticated, it has become possible to detect earlier stages of kidney disease and to differentiate different patterns of protein excretion (1–3). Quantitative immunoassays of selected urinary components such as α1-microglobulin, albumin, IgG, and α2-macroglobulin have been shown to be useful in characterizing the nature of proteinuria (4). Two-dimensional electrophoresis has provided a method for simultaneous analysis of numerous proteins in urine (5, 6). Recently, a new dimension has been added to analysis of urinary components by mass spectrometric techniques, which detect many small peptide components below the size resolution of electrophoresis (7, 8). The highly complex mixtures of small peptides in urine offer the potential for information-rich patterns for clinical diagnosis. Concentrations of urinary peptides serve not only as markers for kidney function but also as markers of other systemic physiologic processes. As examples, immunoassays for specific peptides provide measures of thrombosis and fibrinolysis (9, 10) and endocrine function (11).

In the present study, we sought to identify a simple method to prepare urine specimens for the analysis of small peptide components. Sample preparation represents one of the major challenges for analysis of peptide components in urine specimens by mass spectrometry. Ideally, sample preparation needs to accomplish three tasks: (a) concentration of relatively dilute peptide components; (b) removal of salts that suppress peptide ionization in mass spectrometry; and (c) depletion of albumin and other high-molecular-weight components that comprise most of the total protein mass in urine. Standard methods that have been applied for protein concentration—centrifugal ultrafiltration, acetone precipitation, acid precipitation, dye precipitation, ultracentrifugation, and lyophilization—generally have drawbacks of poor peptide recovery, poorly soluble pellets, or failure to remove salts (6). We examined solid-phase extraction of urinary peptides, using a polymeric sorbent with a pore size that should exclude albumin and other proteins of similar or greater size.

Urine specimens were processed in 6-mL cartridges containing 500 mg of StrataX™ polymeric sorbent (Phenomenex) on a vacuum manifold. Pore size of the sorbent was specified by the manufacturer to be 91 Å, yielding a predicted size exclusion limit of ~20 000 Da. Cartridges were primed with 4 mL of methanol followed by 4 mL of 5 g/L acetic acid before addition of urine specimens, which had been acidified with acetic acid during collection to a pH of 4–5. After extraction of urine, cartridges were washed with 8 mL of 5 g/L acetic acid, and peptides were eluted with 3-mL steps of increasing acetonitrile concentration or with 600 mL/L acetonitrile–5 g/L acetic acid. Measurements of total protein and albumin to determine the amounts of proteins eluted from the cartridges were performed by standard methods (pyrogallol red and immunoturbidimetry, respectively) on a LX-20 analyzer (Beckman-Coulter). C-Peptide was measured by competitive immunoassay with an Immulite 2000 (Diagnostic Products Corp.). Eluates from extraction cartridges were analyzed either after evaporation under nitrogen to ~2 mL or directly.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF) mass spectrometry was performed with an Ultraflex TOF mass spectrometer (Bruker Daltonics) in a linear positive-ion mode. Specimens were applied manually to 384-position target plates at 1-μL aliquots between layers of matrix applied as 1-μL aliquots of 10 g/L sinapinic acid in 750 mL/L acetonitrile–250 mL/L water containing 10 g/L acetic acid. Data were summed for 300 laser pulses collected from 10 positions. Measurements of mass/charge (m/z) were by external calibration. Calibrators and sinapinic acid were purchased from Bruker Daltonics.

Solid-phase extraction of highly proteinuric urine (Table 1) served as good example of the size selectivity and binding capacity of the extraction cartridge. We loaded 10-mL aliquots of urine successively on a single cartridge and analyzed the eluates. The albumin concentrations of flow throughs were approximately the same as the initial
concentrations, indicating very low extraction of albumin. Elution of C-peptide, which has a mass of ~3000 Da, was monitored with a quantitative immunoassay. Flow through concentrations of C-peptide were ~1% of the initial concentration even after loading of 50 mL of the proteinuric specimen, providing evidence that the capacity of the cartridge was not reached even with this large specimen load. Stepwise elution of components bound to the column by increasing concentrations of acetonitrile yielded the largest amounts of C-peptide at 300 mL/L acetonitrile. The C-peptide concentration in the 300 mL/L acetonitrile eluate was 2.4 (0.3)% and 106 (7)%,

Elution of C-peptide, which has a mass of 3000 Da, was

<table>
<thead>
<tr>
<th>Sequential additions</th>
<th>Protein, g/L</th>
<th>Albumin, g/L</th>
<th>C-Peptide, µg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mL of urine</td>
<td>13.5 (96%)</td>
<td>8.7 (99%)</td>
<td>0.55 (1%)</td>
</tr>
<tr>
<td>10 mL of urine</td>
<td>14.1 (101%)</td>
<td>9.0 (102%)</td>
<td>0.87 (2%)</td>
</tr>
<tr>
<td>10 mL of urine</td>
<td>13.9 (99%)</td>
<td>8.7 (99%)</td>
<td>0.62 (1%)</td>
</tr>
<tr>
<td>10 mL of urine</td>
<td>14.1 (101%)</td>
<td>9.0 (102%)</td>
<td>0.55 (1%)</td>
</tr>
<tr>
<td>10 mL of urine</td>
<td>13.8 (99%)</td>
<td>9.0 (102%)</td>
<td>&lt;0.50 (&lt;1%)</td>
</tr>
<tr>
<td>10 mL of 5 g/L acetic acid</td>
<td>0.59 (4%)</td>
<td>0.48 (5%)</td>
<td>&lt;0.50 (&lt;1%)</td>
</tr>
<tr>
<td>3 mL of 100 mL/L acetonitrile</td>
<td>0.39 (3%)</td>
<td>0.02 (0.2%)</td>
<td>0.59 (1%)</td>
</tr>
<tr>
<td>3 mL of 200 mL/L acetonitrile</td>
<td>0.85 (6%)</td>
<td>0.02 (0.2%)</td>
<td>6.6 (12%)</td>
</tr>
<tr>
<td>3 mL of 300 mL/L acetonitrile</td>
<td>3.6 (25%)</td>
<td>0.15 (2%)</td>
<td>662 (1180%)</td>
</tr>
<tr>
<td>3 mL of 400 mL/L acetonitrile</td>
<td>3.1 (22%)</td>
<td>0.23 (3%)</td>
<td>67 (120%)</td>
</tr>
<tr>
<td>3 mL of 500 mL/L acetonitrile</td>
<td>0.33 (2%)</td>
<td>0.02 (0.2%)</td>
<td>3.8 (7%)</td>
</tr>
<tr>
<td>3 mL of 600 mL/L acetonitrile</td>
<td>0.06 (0.4%)</td>
<td>0.002 (0.02%)</td>
<td>3.0 (5%)</td>
</tr>
</tbody>
</table>

a Unprocessed urine contained 14 000 mg/L total protein, 8800 mg/L albumin, and 56 µg/L C-peptide.

Extraction of urine greatly improved the ability to detect peptide components by MALDI (Fig. 1). In the m/z range 1250–5000, few components were apparent in unprocessed urine, and signals were very weak (Fig. 1, top). Analysis of eluates at 400 mL/L acetonitrile showed several components for the proteinuric specimen analyzed in Table 1 (Fig. 1, middle) and for a urine with a protein concentration (70 mg/L) within normal limits (Fig. 1, bottom). The proteinuric specimen had a greater number of components and yielded stronger signals, indicating greater complexity and concentration of peptides. Components detected by mass spectrometry eluted at various acetonitrile concentrations. For the six fractions that were eluted with 100–600 mL/L acetonitrile, >100 different peaks were observed for the proteinuric specimen, and 25 peaks were detected in the specimen with a low protein concentration in the m/z range 1200–7000 (spectra not shown). It is likely that there are thousands of peptide components in urine and that the number observed is likely to depend on the sample preparation and the sensitivity and resolution of the method of analysis (7).

Results of the present study suggest that solid-phase extraction of urine with a polymeric solid phase provides a simple method for extraction of peptides for analysis by mass spectrometry. Peptides were eluted in a salt-free solution containing acetonitrile that might be concentrated further by evaporation. Elution of the greatest number of components at moderate concentrations of acetonitrile (300–400 mL/L) suggests that it should be possible to elute most components off the solid phase. Previously, solid-phase extraction has been applied as a tool for preparation of peptide mixtures, such as tryptic digests of proteins, for MALDI TOF mass spectrometry (12–14). A specialized variation of solid-phase extraction that has been termed surface-enhanced laser desorption/ionization (SELDI) directly extracts peptides onto the target surface for mass spectrometry (15). Typically, solid-phase extraction of peptides has involved use of octadecylsilica as the adsorbent, although some reports note that recovery of hydrophilic peptides can be increased by use of graphite particles or a mixed bed of graphite and octadecylsilica (12–14). Often, sample preparation for MALDI TOF mass spectrometry has been performed in pipette tips packed with tiny amounts of adsorbent. This format provides adequate specimen for MALDI TOF mass spectrometry but not enough for other, traditional clinical laboratory techniques. In addition, octadecylsilica or graphite adsorbents are likely to lack the size selectivity observed in the present experiment because of the larger pore sizes of common adsorbents.

Polymeric adsorbents have potential advantages with respect to capacity and suitability for large volumes of aqueous specimen (12). High capacity is a desirable
characteristic in that it allows concentration of larger volumes of the relatively dilute peptide solutions in urine. The solid-phase extraction procedure described here permits the processing of substantial volumes of specimen and yields eluates of sufficient volume to combine mass spectrometric analysis with traditional clinical laboratory assays that can assess specimen recovery or measure components below the detection limits of mass spectrometry. Size-selective extraction of peptides may be of greatest value for fluids such as proteinuric urine or plasma, which contain high concentrations of albumin or other large proteins. For many diagnostic purposes, it may be useful to combine quantitative assays of specific components such as C-peptide with the high-resolution qualitative analysis provided by MALDI TOF mass spectrometry. The extraction technique here, which is directed at the concentration of peptide and small protein components (<20 000 Da), is complementary to the ultrafiltration techniques that are commonly used in clinical laboratories to concentrate proteins >10 000 Da in urine specimens. The size selectivity of the present extraction technique provides high enrichment of small peptide components such as C-peptide vs a protein such as albumin with a molecular mass of 67 000 Da. In the present study, we did not quantify the amounts of any proteins near the predicted size exclusion limit of ~20 000 Da. Retention of such components is likely to be affected by their molecular shape and to be intermediate in efficiency between small peptides such as C-peptide and proteins the size of albumin.

References
Haptoglobin Phenotypes in Epilepsy, Sayed M.H. Sadrzadeh,* Yasi Saffari, and Jafar Bozorgmehr (Department of Laboratory Medicine, University of Washington, Harborview Medical Center, Seattle, WA 98104; * author for correspondence: fax 206-731-3930, e-mail sadrzade@u.washington.edu)

Seizures occur in ~5% of people, and recur in >20% of that 5% (1, 2). The etiologies of most seizures are unknown, and head trauma is implicated in only 5–10% of cases (3). Blood or blood components, specifically iron, may be etiologically important; intracranial injection of hemoglobin (4), lysed erythrocytes (5), iron-containing proteins (5), or iron salts (6) produced chronic focal spike activity in rodents and cats. Because microhemorrhagic events occur in the central nervous system of all people, inadequate clearance of iron-rich (7) hemoglobin might underlie development of some seizure disorders.

Haptoglobin binds free hemoglobin and removes it from the circulation (8), thus preventing iron loss and kidney damage during hemolysis (9). Haptoglobin contains β- (heavy; 40 kDa) and α- (light; α1 = 8.9 kDa and α2 = 16 kDa) chains. Humans are polymorphic for haptoglobin, with three major phenotypes: Hp 1-1, Hp 2-2, and the heterozygous Hp 2-1 (10). The β-chains are identical in all, with variations dependent on different α-chains. Hp 1-1 expresses only the α1-chain and is the smallest form (86 kDa). Hp 2-1 and Hp 2-2 express α2-chains, which can form polymers of 86–300 kDa (Hp 2-1) and up to 900 kDa (Hp 2-2) (10). Hp 1-1 is biologically the most effective in binding free hemoglobin and suppressing inflammatory responses associated with extracellular (free) hemoglobin (9). In contrast, Hp 2-2 is the least effective (11). The plasma concentrations of haptoglobin are highest in individuals with Hp 1-1 and lowest in those with Hp 2-2, with intermediate concentrations in Hp 2-1 individuals (9).

Haptoglobin also has antioxidant (12), angiogenic (13), and anti-inflammatory effects (11, 14). Furthermore, haptoglobin has a role in regulation of immune responses (15) by suppressing release of cytotoxins from T-helper type 2 cells and regulating T-helper type 1/T-helper type 2 balance (15).

If hemoglobin (or its iron) is involved in the etiology of seizures, then inadequate removal of hemoglobin (by haptoglobin) may be important. We postulated that functional differences between the haptoglobin phenotypes might be related to the severity and frequency of seizure attacks in patients with epilepsy. In this study, we investigated the serum concentrations of haptoglobin and the distribution of haptoglobin phenotypes in people with and without epilepsy and examined the relationship of haptoglobin phenotypes with C-reactive protein (CRP), which, like haptoglobin, is an acute-phase protein.

We studied 92 patients (59 men and 33 women), with a mean age of 43 (range, 21–87) years, who had one or more idiopathic seizures per month and who were treated at our medical center. Controls were 100 volunteers (62 men and 38 women), with a mean age of 44 years. No participants had intravascular hemolysis, liver disease, or trauma. The diagnosis of recurrent idiopathic epilepsy was based on clinical status and electroencephalography results. The study was approved by the local Institutional Review Board.

Phenotyping of haptoglobin was performed by gel electrophoresis followed by peroxidase staining (16). Mobilities of haptoglobin in the samples were compared with authentic samples of Hp 1-1 and Hp 2-2 for phenotype identification. Concentrations of haptoglobin and CRP (17) were measured by fixed-time immunonephelometry with reagents and instrumentation from Dade Behring.

Data are presented as the mean (SE). We used the t-test with Welch’s correction to assess significance of differences of means. Analysis of differences in haptoglobin phenotype distributions in patients and controls was done by χ2 test.

Haptoglobin phenotype 2-2 was significantly associated with recurrent seizures (P <0.001), being present in 67% of the patients and in only 35% of controls. Hp 2-1 and 1-1 were present in 18% and 13% of patients, respectively, and in 50% and 15% of controls. Haptoglobin was undetectable in two patients (2%). The distributions of haptoglobin types were in Hardy–Weinberg equilibrium. The association of Hp 2-2 with seizure attacks persisted when patients were compared with ethnically matched controls (Tables 1 and 2 in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol50/issue6; P <0.05).

Haptoglobin concentrations were significantly higher (P <0.0001) in patients [1.41 (0.08) g/L] than in controls [1.04 (0.04) g/L].

Serum haptoglobin concentrations in patients differed significantly from concentrations in controls when analyzed in relation to their phenotypes (Table 1).

Because haptoglobin is an acute-phase protein, we measured serum CRP in all participants. CRP was significantly higher in patients than in controls [10.1 (1.5) and 1.4 (0.3) mg/L, respectively; P <0.0001]. Not only was pooled serum CRP significantly different in patients vs