Adenosine and Dopamine Simultaneously Determined in Urine by Reversed-Phase HPLC, with On-Line Measurement of Ultraviolet Absorbance and Electrochemical Detection

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This assay method allows the simultaneous determination of adenosine and dopamine in 0.5 mL of human urine within ~35 min, by using isocratic, reversed-phase, high-performance liquid chromatography coupled with an on-line detector for ultraviolet absorbance and electrochemical activity. The analytes were rapidly separated on an affinity column packed with phenylboronate-bonded silica. Mean recoveries for adenosine, 2-chloroadenosine (internal standard), dopamine, and (+)-isoproterenol (also an internal standard) were 91%, 99%, 88%, and 104%, respectively. The calibration curves for adenosine and dopamine were linear (r = 0.999, P <0.01) over the respective concentration ranges of 0.10 to 2.00 mg/L and 0.05 to 1.00 mg/L. Analytical precisions, assessed by the within-day and the day-to-day CVs, were 4.5% and 6.5% for adenosine, and 5.1% and 6.6% for dopamine, respectively. The mean (± SD) concentrations of adenosine (1.27 ± 0.73 mg/L) and dopamine concentrations (0.38 ± 0.25 mg/L) measured in urine from 19 healthy subjects agreed well with those previously reported.

Additional Keyphrases: renal function modulators · solid-phase extraction · phenylboronate affinity column

Intrarenally produced adenosine (Ado) and dopamine (DA) may play a pivotal role in regulating renal hemodynamics and natriuresis. Evidence indicates that Ado and DA have opposite effects, with Ado being vasoconstricting (1) and DA vasodilating on renal vasculatures (2). Previous studies have also shown that administering Ado via the renal artery decreased glomerular filtration rate (GFR) and urinary sodium excretion (1, 3). In contrast, DA has the opposite effect on GFR and urinary sodium excretion (4). Because both of these endogenous substances may be involved in the homeostatic control of renal hemodynamics and sodium disposal, a simultaneous assessment of their intrarenal concentrations under diverse clinical conditions should provide insight into the regulatory mechanisms of the kidneys.

Urinary concentrations of Ado and DA may serve as useful indices of their concentrations in the renal interstitial space or parenchyma. Previous studies have demonstrated that Ado concentrations in urine paralleled those in renal tissue during an ischemic insult (5), and that the administration of dipryridamole, an inhibitor of cellular uptake of Ado, increased urinary concentrations of Ado while reducing GFR (6). Also, a substantial portion of urinary DA is excreted from kidney tissue (7, 8). Thus, a method for simultaneously measuring the concentrations of Ado and DA in urine should have a considerable clinical and research utility. Here, we report the development of a simple method for assay of these endogenous substances in urine by using rapid column chromatography coupled with simple, isocratic, reversed-phase, high-performance liquid chromatography (HPLC) with on-line detection of ultraviolet absorbance and electrochemical activity.

Materials and Methods

Reagents. Ado, guanosine, inosine, cytidine, xanthosine, adenosine 5'-monophosphate (AMP), adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), guanosine 5'-monophosphate (GMP), guanosine 5'-diphosphate (GDP), guanosine 5'-triphosphate (GTP), and inosine 5'-monophosphate (IMP) were purchased from Yamasa Shoyu Co., Choshi, Japan. 5-Bromouridine, 6-chloroguanosine, 5-fluorouridine, 2-chloroadenosine (Cl-Ado), phenylisopropyladenosine, (±)-isoproterenol (IP), sodium 1-octanesulfonic acid used as an ion-pairing agent, dihydroxyphenylalanine, nor-epinephrine, epinephrine, 3,4-dihydroxyphenylacetic acid, DA, and adenosine deaminase (EC 3.5.4.4) were purchased from Sigma Chemical Co., St. Louis, MO. Triethylenamine (used as an amine modifier), methanol, and other reagents of analytical grade were purchased from Wako, Osaka, Japan.

Instrumentation and chromatographic conditions. The analyses were performed with an HPLC system consisting of a Model 100A pump (Altex Scientific, Berkeley, CA), an Eicopack MA-QDS reversed-phase column (250 × 4.6 mm, i.d., packed with 7-µm particles; Eicom Co., Kyoto, Japan), a Model 7125 sample injector (Rheodyne, Berkeley, CA), a Model 8000 ultraviolet absorbance detector (Toyoda Soda Co., Tokyo, Japan) set at 265 nm for detecting Ado and Cl-Ado, and a Model 100 electrochemical detector with a newly developed carbon graphite working electrode WE-3G (Eicom Co.), with the oxidation potential set at 0.7 V vs an Ag/AgCl reference electrode for detecting DA and IP. The HPLC system was assembled so that the analytes separated on the HPLC column flowed first through the ultraviolet absorbance detector, then through the electrochemical detector. Column temperature was maintained at 29 °C with a temperature-controlled water bath.

The mobile phase consisted of an 89/11 (by vol) mixture of methanol with sodium citrate buffer (10 mmol/L, pH 3.1) that contained 10 µmol of EDTA·2Na, 925 µmol of sodium 1-octanesulfonate, and 7.18 mmol of triethylamine per liter. This mobile phase was delivered at a flow rate of 1.0 mL/min. We filtered the mobile phase through a 0.5-µm (pore size) filter membrane (Tosoh Roishi Co., Ltd., Tokyo, Japan) under reduced pressure. All chromatograms were recorded and the areas under the peaks of the respective analytes were integrated with a Model D-2000 Chromato-Integrator (Hitachi Ltd., Tokyo, Japan).

Sample collection and storage. Urine samples were obtained from 19 healthy subjects (ages 25–50 y; 15 men and

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3 Nonstandard abbreviations: Cl-Ado, 2-chloroadenosine; DA, dopamine; IP, (±)-isoproterenol; PBA, phenylboronic acid; and GFR, glomerular filtration rate.

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four women). Freshly voided urine (~10 mL) was collected into a chilled tube containing 26 μmol of sodium metabisulfite, and put on ice. After a brief centrifugation (600 × g, 10 min, 4 °C) to remove insoluble material, urine samples were stored at −80 °C until analyzed.

**Identification of peaks.** Peaks representing Ado and DA in the chromatograms of urine extracts were identified on the basis of their retention times and co-chromatography of the respective authentic standards in several different chromatographic conditions. Identification of the Ado peak was further verified by using adenosine deaminase, which catalyzes the enzymatic conversion of Ado to inosine. A 0.5-mL aliquot of urine incubated with adenosine deaminase, 50 mmol/L, for 60 min at 37 °C was run through the same extraction procedure, as mentioned below, to determine whether this enzymatic treatment for urine had completely eliminated the chromatographic peak representing Ado.

**Sample preparation with phenylboronate (PBA) affinity columns.** For isolating Ado and DA from urine, we used a Bond-Elut column prepacked with 100 mg of PBA-bonded silica (40 μm) in a 1-mL disposable syringe (Analyticchem International, Harbor City, CA) as follows, because this affinity adsorbent has been shown to selectively interact with substances having cis-diol groups (e.g., Ado and DA), particularly under alkaline conditions (9).

Insert the outlet of the column into a chamber connected to a water aspirator. Before applying urine samples on the PBA affinity column, rinse the column with 5 mL of 0.1 mol/L formic acid and then with 5 mL of 0.25 mol/L ammonium acetate (pH 8.8). Mix 0.5 mL of urine with 50 μL of an internal standard solution containing 0.25 μg of IP and 1.00 μg of Cl-Ado in 2.5 mL ammonium acetate buffer (pH 8.8), then apply this to the top of the column, allowing the sample to flow through the column until the liquid meniscus just reaches the top of the column bed. Again, rinse the column with 1 mL of 0.25 mol/L ammonium acetate (pH 8.8), then elute Ado and DA by adding 1 mL of 0.1 mol/L of HCl mixed with methanol (4/1, by vol). Pass this final eluent through a 4.5-μm (pore size) filter membrane (Gelman Science, Tokyo, Japan) and inject 40 μL of the filtrate onto the HPLC column.

**Quantification.** We used the ratios of the area under the peaks of Ado and DA to those of the respective internal standards (i.e., Cl-Ado and IP) to calculate the concentrations of these analytes in each of the urine samples. To prepare the calibration standards, we added known amounts of Ado (i.e., equivalent to the final concentrations of 0, 1.00, and 2.00 mg/L) and of DA (i.e., equivalent to the final concentrations of 0, 0.25, and 0.50 mg/L) into an Ado-free pooled urine, and ran them through the entire procedure along with other samples to be analyzed on each of the assay days.

Ado and DA concentrations in urine are expressed in terms of milligrams per liter and milligrams per gram of creatinine, respectively (to convert SI units: 1 mg of Ado and DA per liter is equivalent to 3.74 and 6.53 μmol/L, respectively). We determined the creatinine concentration in urine by an automated alkaline picrate method, using a SMAC 1 analyzer (Technicon Corp., Tarrytown, NY).

We assessed the absolute recoveries of the analytes extracted from urine by comparing the area under the peaks obtained from standard stock solutions of the analytes with the peak areas from Ado-free urine to which known amounts of the analytes were added to give final concentrations of 1.00, 2.00, and 0.50 mg/L for Ado, Cl-Ado, and IP, respectively. Because endogenous DA is not eliminated from Ado-free urine, we estimated the recovery of DA by comparing the area under the peaks obtained for a stock standard solution with the difference between the area under the peaks obtained for urine samples with and without an added known amount of DA standard (equivalent to 0.25 mg/L).

To examine the linearity of the assay, we added the known amounts of Ado and DA to aliquots of Ado-free urine and determined the peak area ratios of these analytes to the respective internal standards (i.e., Cl-Ado and IP) at eight different concentrations (n = 3 each) from 0 to 2.00 mg/L for Ado and from 0 to 1.00 mg/L for DA.

**Results**

Figure 1 shows representative chromatograms of a urine sample extracted with a PBA affinity column. Under carefully selected chromatographic conditions, the concentrations of Ado, DA, and their respective internal standards in urine can be simultaneously determined within ~35 min by using a simple, isocratic reversed-phase HPLC method. All analytes were well separated from the adjacent, unidentified peaks (Figure 1, A and B). Identification of the Ado peak was further confirmed by the finding that, after a urine sample was treated with adenosine deaminase, the peak thought to represent Ado completely disappeared (Figure 2). In addition, various endogenous nucleosides and catechol analogs that might have been co-extracted with Ado and DA by using the PBA affinity column under the present chromatographic conditions showed no interference with any of the analytes of interest, in terms of their retention times under the chromatographic conditions we used (Table 1).

Among the several compounds examined as possible candidates of internal standards for Ado (5-bromouridine, 6-chloroguanosine, 5-fluorouridine, Cl-Ado, and phenylisopropyladenosine) and DA (IP), Cl-Ado and IP were found most suitable, based on their chromatographic characteristics: phenylisopropyladenosine had a very long retention time.
The mean Ado and DA concentrations in urine from the 19 healthy subjects were 1.27 (SD 0.73) mg/L [or 1.11 (SD 0.48) mg per gram of creatinine] and 0.38 (SD 0.25) mg/L [or 0.33 (SD 0.11) mg per gram of creatinine], respectively, values that agree well with those previously reported (10–13).

Discussion

Despite the growing interest in the physiological roles of Ado and DA in regulating renal hemodynamics and natriuresis (1–6), the development of a specific assay method for urinary Ado has been rather sluggish as compared with DA assay in urine. To our knowledge, ours is the first description of a simple HPLC method for simultaneously determining Ado and DA concentrations in urine, which could be useful as indices of the renal production of these substances (5–8). Several analytical methods have been reported for determining Ado concentrations in certain organs (e.g., heart, brain, and kidney) and in biological fluids other than urine (e.g., myocardial perfusate and plasma) by radioimmunoassay (12), HPLC with fluorometric detection method (14–16), or conventional and microbore HPLC with ultraviolet absorbance detection (17–19). However, none of these methods has been systematically evaluated with respect to their applicability for Ado assay in human urine. Several HPLC methods have been developed for detecting structurally transformed ribosyl purines and pyrimidines (e.g., 1- and 7-methylguanaine, deoxyadenosine) in urine from patients with certain malignancies (9, 20) and those with a severe immunodeficiency syndrome due to an inherited deficiency of adenosine deaminase (10, 21). However, in none of these methods have the assay conditions been optimized for determining Ado in urine. Furthermore, in none of them except one (19) was an internal standard used in the quantification.

Because the boronate selectively interacts with a group of substances having cis-diol structures (e.g., Ado and DA) by forming an anionic complex under alkaline conditions (9), a simple affinity chromatography with immobilized PBA has been shown to offer an attractive alternative to the conventional methods—liquid–liquid extraction and extraction with alumina—for extracting certain nucleosides (9, 20) or catecholamines (22, 23) from biological fluids such as plasma and urine. Most constituents of urine such as nucleic acid bases, deoxyribonucleosides, deoxyribonucleotides, and

Table 1. Retention Times for Urinary Constituents That Might Be Co-Extracted with Ado and DA by the PBA Affinity Column

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time, min&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Compound</th>
<th>Retention time, min&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDP</td>
<td>&lt;2.6</td>
<td>Dihydroxyphenylalanine (DOPA)</td>
<td>9.8</td>
</tr>
<tr>
<td>GTP</td>
<td>&lt;2.6</td>
<td>Norepinephrine</td>
<td>11.4</td>
</tr>
<tr>
<td>ADP</td>
<td>&lt;2.6</td>
<td>Epinephrine</td>
<td>12.9</td>
</tr>
<tr>
<td>ATP</td>
<td>&lt;2.6</td>
<td>3,4-Dihydroxyphenylacetic acid (DOPAC)</td>
<td>16.8</td>
</tr>
<tr>
<td>IDP</td>
<td>&lt;2.6</td>
<td>DA</td>
<td>22.2</td>
</tr>
<tr>
<td>ITP</td>
<td>&lt;2.6</td>
<td>IP (intern. std.)</td>
<td>32.3</td>
</tr>
<tr>
<td>IMP</td>
<td>3.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GMP</td>
<td>3.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMP</td>
<td>4.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inosine</td>
<td>6.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xanthosine</td>
<td>8.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guanosine</td>
<td>8.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytidine</td>
<td>12.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ado</td>
<td>17.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cl-Ado (internal std.)</td>
<td>32.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Measured by ultraviolet absorbance at 265 nm.

<sup>b</sup>Measured by electrochemical detector.
modification in the sample preparation allowed us to inject the eluate from the PBA column directly onto the analytical column, with a substantial savings in total assay time per sample.

We have also established an isocratic, reversed-phase HPLC system, with assay conditions optimized for detecting Ado and DA in urine with suitable internal standards. Simultaneous determination of Ado and DA in such a complex biological material as urine has been made possible by use of a dual detection system: on-line detection of ultraviolet absorbance and electrochemical activity. All nucleosides such as Ado are electrochemically inert at the oxidation potential used in the present study. 0.7 V, so the electrochemical activities of DA and its internal standard, IP, were selectively detected. The composition of the mobile phase (i.e., concentrations of methanol, an ion-pairing agent, and an amine modifier), the pH of the mobile phase, and the column temperature should be carefully selected so as to optimize the assay system for Ado and DA in human urine. The amine modifier, triethylamine, produced a substantial decrease in the tailing of Ado peak, thereby increasing the sensitivity of the current system to Ado. We have also found that Cl-Ado would be a suitable internal standard for the Ado assay in urine in terms of its chromatographic characteristics and affinity to the PBA column (Figure 1 and Table 2). In addition, use of an appropriate internal standard would enable the present method to be more convenient and accurate for determining Ado in urine than the previous methods (9, 20). Under the optimized chromatographic conditions we describe Ado and DA in human urine can be simultaneously determined within ~35 min. We emphasize that this shorter assay time is comparable with that required for determining Ado in urine (9, 20) by using an expensive gradient-HPLC system.

In conclusion: this method allows the simultaneous determination of Ado and DA in a 0.5-mL sample of human urine, and it is simple, rapid, accurate, and inexpensive. We believe that the method has potential value for investigating the physiological roles of these possible endogenous modulators of renal function (1–6) and that it provides researchers with a better chance to examine the interrelations of these substances in various clinical disorders.

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References

Table 2. Analytical Recoveries of Ado, Cl-Ado, DA, and IP from Urine

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc, mg/L</th>
<th>Mean (and SD) recovery, %</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ado</td>
<td>1.00</td>
<td>91.2 (3.9)</td>
<td>4.2</td>
</tr>
<tr>
<td>Cl-Ado</td>
<td>2.00</td>
<td>99.2 (2.6)</td>
<td>2.6</td>
</tr>
<tr>
<td>DA</td>
<td>0.25</td>
<td>87.9 (1.9)</td>
<td>2.2</td>
</tr>
<tr>
<td>IP</td>
<td>0.50</td>
<td>104.1 (5.3)</td>
<td>5.1</td>
</tr>
</tbody>
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n = 8 each.