Simple, Sensitive Assay of Polyamines by High-Performance Liquid Chromatography with Electrochemical Detection after Post-Column Reaction with Immobilized Polyamine Oxidase

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This simple, rapid liquid-chromatographic assay of urinary polyamines (putrescine, spermidine, spermine, and cadaverine) involves electrochemical detection with a post-column immobilized enzyme, polyamine oxidase (EC 1.4.3.6) from soybean seedlings. Polyamines are separated by isocratic ion-pairing reversed-phase chromatography, then enzymatically converted, with release of hydrogen peroxide, via the post-column reactor with immobilized polyamine oxidase; the hydrogen peroxide is detected by electrochemical oxidation on a platinum electrode. The detection limits for injected putrescine, spermidine, and spermine were 0.3, 0.5, 0.6, and 4 pmol, respectively, with linear ranges of two to three orders of magnitude. Reproducibility was also good, with CV values <7%. The efficiency of the immobilized enzyme column was not decreased after analysis of 300 urine samples. Putrescine and spermidine excretion in urine from patients with blood cancers and solid cancers was significantly increased.

Additional Keyphrases: chromatography, reversed-phase, cancer, immobilized enzyme, urine.

Since the first report by Russell (1) of increased urinary excretion of polyamines by patients with various types of cancers, many techniques and procedures have been reported for assay of polyamines in urine and serum, including high-voltage paper electrophoresis, analysis by an amino acid analyzer, and "high-performance" liquid chromatography (HPLC) (2).

Polyamines have usually been quantified by HPLC, with pre-column or post-column derivatization by benzoyl chloride (3) for measuring ultraviolet absorbance, dabeyl chloride (4) for measuring visible absorbance, or dansyl chloride (5) or o-phthaldehyde (6) for fluorescence detection. These derivatizations are tedious, and not necessarily specific.

We describe here our sensitive (sub-picomole), selective analytical method for quantifying polyamines after HPLC by use of post-column generation of hydrogen peroxide by use of immobilized polyamine oxidase (EC 1.4.3.6) from soybean seedlings and subsequent electrochemical oxidation.

Materials and Methods

Apparatus. We used a computer-controlled pump (CCFM; Bioanalytical Systems, West Lafayette, IN), a 6 × 150 mm column of polymer-based C₁₈ Biophase-III (BAS Co., Tokyo, Japan), a sample injector with a 50-μL loop (Model 7125; Rheodyne, Cotati, CA), and an amperometric detector (LC-4B; Bioanalytical Systems) with a platinum working electrode. A 4 × 20 mm stainless-steel column of immobilized enzyme was placed between the analytical column and the detector. The enzyme column was stored at 4 °C at the end of every working day.

Enzyme-immobilized column. Polyamine oxidase was purified from soybean seedlings as described previously (7). The enzyme was immobilized on aminopropyl-derivatized controlled-pore glass beads (Aminopropyl-CPG, 200–400 mesh, pore size 40 nm; Electro Nucleonics, Fairfield, NJ) by a coupling reaction with glutaraldehyde. Twenty-five milligrams of the enzyme (30 kU/g) was dissolved in 10 mL of phosphate buffer (0.1 mol/L, pH 6.8). About 500 mg of aminopropyl-CPG activated with glutaraldehyde was added to this solution, and the mixture was allowed to stand for 24 h. The immobilized enzyme beads (light brown in color) were filtered, washed with water, and then packed by pouring, with tapping, into a stainless-steel column filled with water.

Reagents. Hydrochloride salts of putrescine (Put), spermidine (Spd), spermine (Spm), and cadaverine (Cad) were obtained from Sigma Chemical Co., St. Louis, MO 63178.⁷ Sodium 1-octanesulfonate for ion-pair chromatography was purchased from Tokyo Kasei Co., Tokyo, Japan. All other chemicals (analytical grade) were obtained from Wako Pure Chemicals, Osaka, Japan.

Standards. We prepared 1 mmol/L stock solutions of the polyamines in 0.1 mol/L perchloric acid, and stored them at −20 °C. Each day, we prepared the working standard solution of polyamines (Put, 10 pmol/L; Cad, 25 pmol/L; Spd, 30 pmol/L; Spm, 40 pmol/L) from the stock solutions.

Sample preparation. Untimed urine samples were collected before breakfast at room temperature from 79 normal subjects and from 91 patients with various types of cancer. An aliquot of each urine was stored frozen until analysis.

After thawing the samples, we mixed 1.0 mL of urine and 1.0 mL of 6 mol/L HCl in a 13 × 100 mm screw-capped tube and hydrolyzed for 3 h in boiling water. The hydrolysate was neutralized with 1 mol/L NaOH, then diluted with 20 mL of 5 g/L sodium 1-octanesulfonate reagent. We applied the mixture to a Sep-Pak C₁₈ column (Waters, Milford, MA). After the column drained, we washed it with 50 mL of water, then eluted the polyamines with 0.5 mL of 0.5 mol/L KH₂PO₄. The eluate was centrifuged at 8000 × g in a Microfuge B (Beckman Instruments, Inc., Palo Alto, CA), and 10 μL of the

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⁷ Nonstandard abbreviations: Put, putrescine; Spd, spermidine; Spm, spermine; Cad, cadaverine; and Cr, creatinine.

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supernate was injected into the chromatograph.

**Chromatographic conditions.** The flow rate was 0.4 mL/min, and the detector potential was +500 mV vs an Ag/AgCl reference electrode at 20 nA full-scale. To prepare the mobile phase, we dissolved, in 1 L of water purified with a Milli Q system (Millipore Corp.), 31.35 g of K₂HPO₄, 2.7 g of KH₂PO₄, and 500 mg of sodium 1-octanesulfonate (pH 7.7). The solution was filtered through a 0.22-μm (pore-size) filter, and de-gassed before use.

**Creatinine determination.** Creatinine (Cr) concentrations in urine were determined by the method of Bonsnes and Taussky (8), and the concentration of polyamines was expressed as nanomoles per milligram of creatinine.

**Statistical analysis.** We used the two-tailed Student’s *t*-test for independent samples to evaluate significant differences between normal subjects and patients with cancer.

**Results and Discussion**

To separate polyamines by isocratic ion-pairing reversed-phase chromatography with a low salt eluent in a reasonably short time, we used a new, polymer-based, reversed-phase column that can withstand an alkaline mobile phase. Polyamine oxidase immobilized in a post-column reactor specifically oxidizes polyamines, leading to the formation of hydrogen peroxide, as illustrated here for Spd:

\[
\text{NH}_2\text{CH}_2\text{CH(NH}_3\text{)}_2\text{H}_2\text{O} + \text{O}_2 \rightarrow \\
\text{OHC(CH}_2\text{)}_2\text{CH(NH}_3\text{)}_2\text{H}_2\text{O} + \text{H}_2\text{O}_2
\]

The enzyme has a wide substrate specificity and oxidizes

Put, Spd, Spm, and Cad. The hydrogen peroxide produced can be oxidized electrochemically on a platinum electrode.

Figure 1 shows chromatograms obtained for the standard solution and a sample of urine from a normal subject. Before HPLC, the urine was hydrolyzed and pre-treated as described above. A complete chromatographic run took 40 min.

Mean analytical recoveries of Put, Cad, Spd, and Spm were 75.4% (SD 1.6%), 96.5% (SD 3.8%), 101.2% (SD 5.2%), and 84.1% (SD 5.3%), respectively. Standard curves for the polyamines are shown in Figure 2. The dynamic ranges of peak responses were linear over two to three orders of magnitude. Lower limits of sensitivity (signal/noise ratio of 10) were 0.3, 0.5, 0.6, and 4 pmol injected for Put, Cad, Spd, and Spm, respectively. Table 1 summarizes the results of

<table>
<thead>
<tr>
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<th>Mean (and SD) concn, pmol</th>
<th>CV, %</th>
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<tbody>
<tr>
<td>Put</td>
<td>Within-run 21.0 (0.45)</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>Between-run 19.8 (1.23)</td>
<td>6.2</td>
</tr>
<tr>
<td>Cad</td>
<td>Within-run 50.8 (1.08)</td>
<td>2.1</td>
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<tr>
<td></td>
<td>Between-run 48.6 (2.69)</td>
<td>5.5</td>
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<tr>
<td>Spd</td>
<td>Within-run 9.6 (0.49)</td>
<td>5.1</td>
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<tr>
<td></td>
<td>Between-run 9.0 (0.33)</td>
<td>3.7</td>
</tr>
<tr>
<td>Spm</td>
<td>Within-run 7.3 (0.46)</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>Between-run 6.9 (0.48)</td>
<td>6.9</td>
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*Fig 2. Standard curve for quantification of polyamines by the present method.*

*Fig 1. Chromatograms obtained for (a) a standard solution containing (1) Put, 10 pmol; (2) Cad, 25 pmol; (3) Spd, 30 pmol; (4) Spm, 40 pmol; and (b) a urine sample containing (1) Put, 7.5 nmol/mg Cr; (2) Cad, 14.2 nmol/mg Cr; (3) Spd, 57.3 nmol/mg Cr; and (4) Spm, 16 nmol/mg Cr. H, histamine; U, unknown peak.*
within-run and between-run precision studies.

Durability of the immobilized enzyme column was as follows: we observed no loss of the enzyme activity during continual use for at least four months during which about 300 urine samples were analyzed.

Some amine compounds besides polyamines in urine may be oxidized by the polyamine oxidase, but no unidentified peaks overlapped any of the polyamine peaks in the chromatograms.

We used this method to determine polyamines in urines from normal control subjects and from patients with blood cancers and solid cancers. Put and Spd were increased significantly in patients with blood cancers and solid cancers, whereas Spm was increased only slightly (Table 2).

References