A New Simple Enzymatic Assay Method for Urinary Polyamines in Humans

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ABSTRACT

We developed a new simple enzymatic assay method for measuring urinary polyamines (total amount of putrescine, spermidine, and cadaverine), using an acylpolyamine amidohydrolase and a putrescine oxidase. First, conjugated polyamines (putrescine, spermidine, and cadaverine) in urine were hydrolyzed by incubation with an acylpolyamine amidohydrolase at 30°C for 1 hr. Then, free polyamines were separated by cation-exchange chromatography and incubated with a putrescine oxidase at 30°C for 30 min. Hydrogen peroxide formed in this reaction was measured spectrophotometrically (at 514 nm).

Polyamine levels in urine were determined in 70 normal subjects, 124 patients with cancer, and 52 patients with diseases other than cancer. Elevation above 3 S.D.s of the normal mean was found in 90 (72.6%) of the 124 patients with cancer and in 6 (11.5%) of the 52 patients with diseases other than cancer. Serial studies in 19 patients with cancer indicated that polyamines in urine were reduced after successful surgery.

Our new method is simple and rapid and therefore very useful for routine clinical application. Moreover, our data indicate that the determination of polyamine levels is useful as a marker of disease activity in patients with cancer.

INTRODUCTION

An increased amount of polyamines in the urine of human cancer patients was first reported in 1971 (14). Since then, there have been many reports describing the estimation of polyamines in the urine from patients with cancer (5, 7, 11, 13, 18). Also, many methods for measuring polyamines have been developed as reviewed by Bachrach (2), Russell and Durie (13), and Seiler (15), using thin-layer chromatography, gas chromatography, amino acid analyzers, and HPLC.3 Radioimmunoassays (3, 4) and, recently, an enzymatic assay method (8) have also been reported. However, many of the methods are cumbersome and tedious and require special equipment.

Polyamines in the urine are mainly in a conjugated form (1, 12, 14, 18), so acid hydrolysis is necessary before analysis to release free polyamines. Many methods involve acid hydrolysis of the urine, which is cumbersome and time consuming (6 N HCl, 110°C, 6 to 18 hr) (8, 13, 14). Clinical application requires the development of a more simple and rapid method. Our present method using an acylpolyamine amidohydrolase, which hydrolyzes conjugated polyamines (putrescine, spermidine, and cadaverine) in the urine, and a putrescine oxidase, which oxidizes free polyamines, is simple and rapid. Therefore, this method seems to be very useful for routine clinical application.

MATERIALS AND METHODS

Chemicals. Putrescine dihydrochloride, cadaverine dihydrochloride, and spermidine trihydrochloride were purchased from Sigma Chemical Co., St. Louis, Mo.; horseradish peroxidase (type II) was from Boehringer, Mannheim, Germany; Bio-Rex 70 (100 to 200 mesh) was from Bio-Rad Laboratories, Richmond, Calif.; and 4-aminoantipyrine and 2,3-dichlorophenol were from Nakarai Chemicals, Ltd., Kyoto, Japan. All other chemicals were of analytical grade. The acetylated polyamines were prepared according to published procedures (17).

Preparation of Bio-Rex 70 Columns. Fifty g of Bio-Rex 70 resin were stirred in 500 ml of distilled water for 5 min and allowed to settle for 5 min. The supernatant was removed by decantation. This washing procedure was repeated 5 times. The resin was then suspended in 450 ml of distilled water and stirred for 30 min. Then, 2 ml of the resin were packed into 5 ml disposable syringes.

Creatinine Determination. Urinary creatinine concentrations were determined by the method of Jaffé (6), and creatinine excretion (g/24 hr) was calculated.

Putrescine Oxidase. Putrescine oxidase was purified from Micrococcus rubens cells as described previously (9). We used a partially purified enzyme for practical use. Partial purification of this enzyme was performed as follows. The cells (560 g) were suspended in 1500 ml of 10 mM phosphate buffer (pH 7.2) and then passed twice through a Dyno-Mill homogenizer at 3000 rpm at a rate of 2 liters/hr. The supernatant was pooled and precipitated by 40 to 70% saturation with ammonium sulfate. The precipitate was dissolved in 200 ml of 5 mM phosphate buffer (pH 7.2) and dialyzed against the same buffer overnight. Then, the enzyme solution was adsorbed on a DEAE-cellulose column (5 x 50 cm) equilibrated with 10 mM phosphate buffer (pH 7.2) and eluted with a linear gradient of NaCl from 0.1 to 0.4 M in a total volume of 3000 ml. The active fractions were pooled and used as the partially purified enzyme (20 units/ml). This enzyme was specific for putrescine, spermidine, and cadaverine (10).

Preparation of Acylpolyamine Amidohydrolase. Cells of Streptomyces averellus R-20 (1.5 kg) were suspended in 6 liters of 50% ethanol buffer composed of 3 liters of ethanol and 3 liters of 5 mM phosphate buffer (pH 7.2). The suspension was passed twice through a Dyno-Mill Model KDL cell rupture instrument at a rate of 60 ml/min. The homogenate was centrifuged at 15,000 rpm for 30 min, and the supernatant was mixed with 1.5 liters of DEAE-cellulose for 1 hr. The DEAE-cellulose which adsorbed the enzyme was filtered on a Buchner funnel and washed with 10 liters of 5 mM phosphate buffer (pH 7.2). The washed DEAE-cellulose was packed into a column (9 x 74 cm), and the column was washed with 5 liters of 5 mM phosphate buffer supplemented with 25 mM ammonium sulfate. The enzyme was eluted with 5 mM phosphate buffer supplemented with 150 mM ammonium sulfate. The active fractions were concentrated and desalted by ultrafiltration. Subsequently, the crude enzyme was applied on a DEAE-cellulose column (5.5 x 28 cm) equilibrated with 5 mM phosphate buffer (pH 7.2) and eluted with a linear gradient of ammonium sulfate from 50 to 80 mM in a total volume of 5 liters of 5 mM phosphate buffer (pH 7.2). The active fractions were pooled and concentrated to 100 units/ml. This enzyme was purified 170-fold to give a specific activity of 35 µmol/mg/min when 1 mM acetylputrescine was used as a substrate. This enzyme solution was stored at -20°C until use and found to be stable for more than 1 month. This enzyme solution was incubated at various pHs with each synthetic acetylpolyamine (putrescine, spermidine, and cadaverine), and...
each free polyamine liberated was measured using putrescine oxidase as described previously (9, 10). The pH optimum of this enzyme was 8.0, and it was active toward acetylpolymamines (putrescine, spermidine, and cadaverine). The details of the purification and characterization of this enzyme will be described elsewhere.

Acid Hydrolysis of Urine. Urine aliquots (3 ml) were placed in test tubes (1.5 x 16.5 cm). To the test tubes, 3 ml of concentrated hydrochloric acid were added, and the mixtures were heated at 100° for 12 hr in an oil bath. The hydrolysates were cooled and centrifuged at 3000 rpm for 5 min. The supernatants were evaporated to dryness. The residues were dissolved in 5 ml of 0.2 M Tris-HCl buffer (pH 9.0) and loaded on columns (0.2 ml) of Bio-Rex 70. The columns were washed with 10 ml of distilled water, and polyamines were eluted with 3 ml of 0.2 M trichloroacetic acid. The eluates were used as HPLC samples.

Polyamine Analysis by HPLC. HPLC was performed with a system composed of a Model SJ-1700 autosampler, a Model 1700 autoinjector (Atto Co., Tokyo, Japan), a Model SGR-1A solvent step-gradient programmer, a Model FLD-1 fluorescence detector, a Model LC-3A pump unit, and a Model C-R1A chart recorder (Shimadzu Seisakusho, Kyoto, Japan). Separation of the polyamines was carried out on a column (25 cm x 4 mm) packed with Hitachi custom ion-exchange resin, No. 2613 (Mitsubishi Chemical Industries, Ltd., Japan), at 50° with sodium citrate (0.018 M), sodium chloride (0.4%), methanol (4.0%), Brij-35 (0.016%), n-caproic acid (0.007%), and trichloroacetic acid. The retention times for putrescine, spermidine, and cadaverine were 18.2, 21.6, and 27.4 min, respectively. The column was regenerated with 0.2 M sodium hydroxide for 2 min and equilibrated with Buffer A for 15 min. Polyamines were detected by monitoring fluorescence intensity generated by the reaction between the column eluent and o-phthalaldehyde reagent which was prepared by dissolving 25.2 g of boric acid, 12.8 g of sodium hydroxide, 1.0 g of Brij-35, 2 ml of 2-mercaptoethanol, and 800 mg of o-phthalaldehyde dissolved in 12 ml of ethanol in 1 liter of distilled water. The column eluent and o-phthalaldehyde were mixed at a 1:1 ratio at 50°.

Urine Samples. Urine specimens (24 hr) were obtained from 70 normal subjects; 105 patients with advanced cancer (stomach (48), colon (25), lung (19), esophagus (6), gall bladder (3), pancreas (2), and liver (2)); 19 patients with hematological cancers [acute lymphocytic leukemia (8), acute myelogenous leukemia (4), chronic myelogenous leukemia (1), and multiple myeloma (6)]; and 52 patients with diseases other than cancer. After the 24-hr collection was completed, the specimens were frozen at −20° until the analysis was performed. NaN₃ (0.01%, w/v) was used as a preservative during collection and storage. All specimens were obtained prior to therapy. Collections from some patients were repeated after successful surgical removal of tumors.

Enzymatic Assay for Urinary Polyamines. First, 3 ml of urine samples were adjusted to pH 8.0 with 2 ml of 0.5 M Tris-HCl buffer (pH 8.0) and incubated with acetylpolymamine amidohydrolase (50 units), which hydrolyzes conjugated polyamines (putrescine, spermidine, and cadaverine) at 30° for 1 hr. After centrifugation at 3000 rpm for 10 min, the supernatant was loaded on a column (0.2 ml) of Bio-Rex 70. The column was washed with 10 ml of distilled water and then eluted with 3 ml of 0.2 M trichloroacetic acid. The eluate was adjusted to pH 7.5 with 0.9 ml of 0.2 M Tris-HCl buffer and further eluted with 3 ml of 0.5 M Tris solution and incubated with a 3-ml assay mixture which contained 2.67 ml of 0.1 M Tris-HCl buffer (pH 8.0), 30 μl of 0.03 M dichlorophenol in 40% ethanol, 30 μl of 0.05 M 4-aminopyrine in water, 120 μl of peroxidase solution (1.0 mg/ml), and 150 μl of putrescine oxidase solution (20 units/ml) at 30° for 30 min. Hydrogen peroxide formed in this reaction was measured spectrophotometrically (514 nm) (absorbance thus obtained, Ab). The blank test differed in the respect that 3 ml of distilled water were added in place of the urine (absorbance obtained, Aα). As an external standard, 150 μl of 0.5 M putrescine in distilled water and 2.85 ml of distilled water were added in place of the urine (absorbance obtained, Aα). The calculation was done as follows:

\[
\text{HPLC}_{\text{polyamines}}(\text{nmol}) = \frac{(A - A_\alpha) \times 24\text{-hr urine volume (ml)}}{A_e - A_\alpha}
\]

RESULTS

Sensitivity and Specificity. The relationship between the absorbance resulting from the enzyme reaction and various amounts of acetylpolymamines was found to be about 10 nmol for each of acetylputrescine, acetylspermidine, and acetylcadaverine. We also obtained the same results when different amounts of acetylpolymamines were added to 3 ml of normal urine (data not shown).

The specificity of this method is dependent on the substrate specificity of putrescine oxidase. To check the specificity of the present method, various amines were tested. As described previously (10), the putrescine oxidase was specific for putrescine, spermidine, and cadaverine. Other amines tested including spermine, ethylenediamine, 1,3-propane diamine, 1,6-hexanedi-amine, arginine, butyline, octopamine, serotonin, tryptamine, benzylamine, tyramine, and synephrine were attacked negligibly.

Therefore, the present method is considered to measure the total amount of putrescine, spermidine, and cadaverine, specifically.

Recovery and Precision. Acetylputrescine, acetylspermidine, and acetylcadaverine, 100 nmol and 300 nmol of each, were added to 3 ml of urine. The recoveries of acetylputrescine, acetylspermidine, and acetylcadaverine for 100 nmol of each were calculated to be 92.4%, 87.9%, and 91.0%, respectively,

\[
\text{HPLC}_{\text{polyamines}}(\text{nmol}) = \frac{(A - A_\alpha) \times 24\text{-hr urine volume (ml)}}{A_e - A_\alpha}
\]

Chart 1. The relationship between the absorbance resulting from the enzyme reaction and acetylpolymamine concentrations. Various amounts of acetylpolymamines (0 to 300 nmol) were added to 3 ml of distilled water and assayed by the enzymatic method as described under "Materials and Methods." □, acetylputrescine; ●, acetylspermidine; △, acetylcadaverine.
and for 300 nmol of each were 93.3%, 89.8%, and 92.2%, respectively.

Using 3 different samples of urine, the precision of the assay was evaluated by assaying 10 times in one assay (intraassay) or in duplicate in 10 consecutive assays (interassay). The results are shown in Table 1. The intraassay variance was less than 4%, and the interassay variance ranged between 5 and 8%.

Comparison of Acid Hydrolysis and Enzymatic Hydrolysis. To evaluate the validity of enzymatic hydrolysis, we compared polyamine concentrations in urine from 39 normal subjects and 23 patients with cancer after acid hydrolysis (6 N HCl, 12 hr, 100°C) and enzymatic hydrolysis using acylpolyamine amidohydrolase as described under "Materials and Methods." After acid hydrolysis or enzymatic hydrolysis, the total amount of putrescine, spermidine, and cadaverine was determined by HPLC as described under "Materials and Methods." As shown in Chart 2, the results obtained for enzymatic hydrolysis correlated well with those for acid hydrolysis ($r = 0.971$, $y = 1.016 x + 0.531$, $n = 62$).

Comparison of Enzymatic Assay and HPLC Assay. To evaluate the validity of the putrescine oxidase assay, we compared polyamine concentrations in urine from 57 normal subjects and 26 patients with cancer by both the putrescine oxidase assay and HPLC assay as described under "Materials and Methods." After acid hydrolysis (6 N HCl, 100°C, 12 hr) as described under "Materials and Methods," the total amount of putrescine, spermidine, and cadaverine was determined by the putrescine oxidase assay and HPLC assay. As shown in Chart 3, there was a good correlation between the 2 methods. The regression equation was $y = 0.841 x + 1.786$, and the correlation coefficient was 0.989.

Application of the Method to Urinary Polyamines in Cancer Patients. Twenty-four-hr urine collections were obtained from 70 normal subjects. The mean level of urinary polyamines (total amount of putrescine, spermidine, and cadaverine) in urine from 57 normal subjects (•) and 26 patients with cancer (C) were determined by the putrescine oxidase assay and HPLC assay after acid (HCl) hydrolysis. The regression equation was $y = 0.841 x + 1.786$, and the correlation coefficient was 0.989.

Chart 2. Correlation between enzymatic hydrolysis and acid (HCl) hydrolysis. Polyamine concentrations (μM) (total amount of putrescine, spermidine, and cadaverine) in urine from 39 normal subjects (•) and 23 patients with cancer (C) were determined by HPLC after enzymatic hydrolysis or acid (HCl) hydrolysis. The regression equation was $y = 1.016 x + 0.531$, and the correlation coefficient was 0.971.

Chart 3. Correlation between the putrescine oxidase assay and HPLC assay. Polyamine concentrations (μM) (total amount of putrescine, spermidine, and cadaverine) in urine from 57 normal subjects (•) and 26 patients with cancer (C) were determined by the putrescine oxidase assay and HPLC assay after acid (HCl) hydrolysis. The regression equation was $y = 0.841 x + 1.786$, and the correlation coefficient was 0.989.

Table 1

<table>
<thead>
<tr>
<th>No. of assays</th>
<th>[Polyamine] (μM)</th>
<th>Coefficient of variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraassay sample</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>21.3 ± 0.76a</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>50.8 ± 1.90</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>95.4 ± 3.72</td>
</tr>
<tr>
<td>Interassay sample</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>19.2 ± 0.95</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>51.3 ± 3.33</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>98.7 ± 7.80</td>
</tr>
</tbody>
</table>

* Mean ± S.D.
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Table 2

<table>
<thead>
<tr>
<th>Case</th>
<th>Diagnosis</th>
<th>Preoperative</th>
<th>Postoperative</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Stomach cancer</td>
<td>158.5</td>
<td>39.7</td>
</tr>
<tr>
<td>2</td>
<td>Stomach cancer</td>
<td>103.3</td>
<td>21.3</td>
</tr>
<tr>
<td>3</td>
<td>Stomach cancer</td>
<td>356.8</td>
<td>37.5</td>
</tr>
<tr>
<td>4</td>
<td>Stomach cancer</td>
<td>214.3</td>
<td>36.4</td>
</tr>
<tr>
<td>5</td>
<td>Stomach cancer</td>
<td>100.5</td>
<td>25.0</td>
</tr>
<tr>
<td>6</td>
<td>Stomach cancer</td>
<td>155.1</td>
<td>31.2</td>
</tr>
<tr>
<td>7</td>
<td>Stomach cancer</td>
<td>120.4</td>
<td>27.9</td>
</tr>
<tr>
<td>8</td>
<td>Stomach cancer</td>
<td>77.9</td>
<td>35.8</td>
</tr>
<tr>
<td>9</td>
<td>Stomach cancer</td>
<td>57.6</td>
<td>36.7</td>
</tr>
<tr>
<td>10</td>
<td>Stomach cancer</td>
<td>128.2</td>
<td>43.5</td>
</tr>
<tr>
<td>11</td>
<td>Stomach cancer</td>
<td>82.3</td>
<td>19.6</td>
</tr>
<tr>
<td>12</td>
<td>Stomach cancer</td>
<td>63.4</td>
<td>22.3</td>
</tr>
<tr>
<td>13</td>
<td>Colon cancer</td>
<td>50.4</td>
<td>35.9</td>
</tr>
<tr>
<td>14</td>
<td>Colon cancer</td>
<td>90.0</td>
<td>37.8</td>
</tr>
<tr>
<td>15</td>
<td>Colon cancer</td>
<td>69.3</td>
<td>33.1</td>
</tr>
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<td>16</td>
<td>Colon cancer</td>
<td>91.1</td>
<td>35.1</td>
</tr>
<tr>
<td>17</td>
<td>Colon cancer</td>
<td>89.6</td>
<td>30.7</td>
</tr>
<tr>
<td>18</td>
<td>Colon cancer</td>
<td>48.7</td>
<td>25.1</td>
</tr>
<tr>
<td>19</td>
<td>Colon cancer</td>
<td>131.4</td>
<td>41.9</td>
</tr>
</tbody>
</table>

The value of polyamines (putrescine + spermidine + cadaverine) was expressed as µmol per g creatinine per 24-hr excretion.

Samples were collected a week before surgery.

Samples were collected 3 weeks after surgery.

DISCUSSION

Many methods for measuring polyamines have been developed, as reviewed by Bachrach (2), Russell and Durie (13), and Seller (15), using thin-layer chromatography, gas chromatography, amino acid analyzers, HPLC, and radioimmunoassays. Thin-layer chromatography, which is based on the fluorometric assay of dansyl derivatives, is sensitive and versatile. However, there are some disadvantages. Before analysis, time-consuming and laborious acid hydrolysis is necessary to release free polyamines from the conjugates. As far as screening purposes are concerned, this method cannot be automated. Side products of the derivative-forming reaction create problems; therefore, it is necessary to remove a large proportion of the side products by prechromatography on columns containing silica gel.

The automated amino acid analyzer method is fairly specific and moderately sensitive, but the major disadvantages of this approach are the high cost of the instrument and its maintenance. Besides these disadvantages, the time required for separation and column regeneration for a single sample is approximately 60 min. Therefore, the maximum number of samples that can be analyzed per day is 24. Moreover, before analysis, time-consuming and laborious acid hydrolysis is necessary. Thus, this method is not suitable for routine clinical application.

Gas chromatography, especially the gas chromatography-mass spectrometry technique utilizing deuterated analogues of polyamines as internal standards, is sensitive. However, before analysis, time-consuming and laborious acid hydrolysis is necessary. Furthermore, the separation time for a single sample is 30 to 60 min. Therefore, the maximum number of samples that can be analyzed per day is less than 50. Thus, this method is also not suitable for routine clinical application.

HPLC is a potentially useful alternative to thin-layer chromatography and amino acid analyzers. This method is sensitive, but before analysis, time-consuming and laborious acid hydrolysis is necessary. Furthermore, the separation time for a single sample is 30 to 60 min. Therefore, the maximum number of samples that can be analyzed per day is less than 50. Thus, this method is not suitable for routine clinical application.

A radioimmunoassay involving the use of anti-spermine antibody was reported (3). However, this anti-spermine antibody cross-reacts with spermidine (22%). From the point of specificity, this reported radioimmunoassay was not suitable for precise analytical purposes. Bartos et al. (4) subsequently developed a radioimmunoassay involving the use of specific anti-spermidine antibody. However, this radioimmunoassay has not been applied to clinical studies. The radioimmunoassay will be a useful tool for clinical application due to its sensitivity and simplicity, when specific antibodies for each of the polyamines are produced. However, to our knowledge, specific anti-spermine, anti-cadaverine, and anti-putrescine antibodies have not been reported. It also remains to be seen whether the radioimmunoassay can differentiate between free and conjugated forms of polyamines.

Recently, Matsumoto et al. (6) developed an enzymatic assay method for measuring the total amount of putrescine and cadaverine in urine using human placental diamine oxidase. However, their method has 2 disadvantages. One is that it involves acid hydrolysis (2 N HCl, 100°, 6 hr) which is time-consuming and
laborious. The other is that their method is unable to measure spermidine which is usually increased in the urine of cancer patients (13).

Our present method for measuring the total amount of putrescine, spermidine, and cadaverine in urine has many advantages compared with the above methods. Our method does not require special equipment, and the technique is simple. In our method, the laborious and time-consuming (6 to 18 hr) acid hydrolysis step is replaced by enzymatic hydrolysis. By using this enzymatic hydrolysis, we can hydrolyze conjugated polyamines in urine in only 1 hr. Moreover, this step is very simple. This simple and rapid hydrolysis is of great advantage. Furthermore, it is confirmed that urinary conjugated polyamines were completely hydrolyzed by the enzymatic hydrolysis as shown in Chart 2. After hydrolyzing conjugated polyamines, the total amount of putrescine, spermidine, and cadaverine was measured enzymatically using putrescine oxidase. This step is also simple and rapid (within 1 hr) and is validated in that good correlation was obtained between the enzymatic assay and HPLC assay as shown in Chart 3. With this enzymatic assay, we can measure the total amount of putrescine, spermidine, and cadaverine but cannot measure individual polyamines in urine. As far as clinical studies are concerned, our method for measuring the total amount of putrescine, spermidine, and cadaverine is advantageous in the respect that any type of elevation of polyamines can be detected. As described by Lipton et al. (7), the total polyamine level and elevations of 2 or more of the individual polyamines appear to be the most reliable indexes of the presence of cancer. By our method, 100 or more assays can be conducted within 3 hr by one technician.

Elevated levels of urinary polyamines were found in 90 (72.6%) of 124 patients with various cancers by our method. The high incidence of increased excretion of polyamines was also reported by other investigators using different analytical methods (5, 7, 18).

Matsumoto et al. (8) have described recently an enzymatic assay method for measuring the total amount of putrescine and cadaverine in urine. They reported that the mean level in normal subjects was 21.4 µmol/g creatinine and that elevated levels of polyamines were found in about 70% of patients with stomach cancer and in about 70% of patients with colon cancer, although data of their patients were preliminary. Our data showing that the mean level of total urinary polyamines in normal subjects was 20.0 ± 8.3 µmol/g creatinine and that elevation of total urinary polyamines was found in 72.6% of patients with various cancers were not inconsistent with their data.

In summary, we have established a new simple enzymatic assay method for total urinary polyamines. Our method seems to be superior to other methods with respect to the simplicity, rapidity, and applicability to routine analysis of urinary polyamines as an aid for the diagnosis of cancer and in monitoring the response to therapy for cancer.

ACKNOWLEDGMENTS

We wish to thank Professor T. Wada and Dr. Z. Yamasaki, the Department of Surgery, Faculty of Medicine, University of Tokyo, for providing the patients for this study. We are also grateful to Professor F. Takaku, the Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, for the critical reading of the manuscript.

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