Melanoma Detection by Enzyme-Radioimmunoassay of L-Dopa, Dopamine, and 3-O-Methyldopamine in Urine

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This enzyme-radioimmunoassay for the measurement of L-dopa, dopamine, and 3-O-methyldopamine is based on the incubation of urine in the presence of catechol-O-methyltransferase, aromatic-L-amino-acid decarboxylase, and S-adenosylmethionine. The O-methylated dopamine metabolite formed, 3-O-methyl-dopamine, was characterized by radioimmunoassay. To evaluate the role of L-dopa metabolism in melanoma, we used the enzyme-radioimmunoassay to assess concentrations of L-dopa, dopamine, and 3-O-methyldopamine in urine from 10 healthy subjects, 10 hospitalized patients without melanoma, and 28 patients with different degrees of melanoma. The effect of surgery for melanoma on urinary output of these catechols of melanoma patients was also evaluated. No significant difference in urinary L-dopa, dopamine, and 3-O-methyldopamine excretion rates was seen between normal subjects (L-dopa 1.3 ± 0.3, dopamine 147 ± 38, and 3-O-methyldopamine 31.4 ± 13.6 μg/24 h), hospitalized patients without melanoma, and amelanotic melanoma patients. However, the excretion rates for these metabolites in melaninocytic melanoma (L-dopa 5.6 ± 1.2, dopamine 555 ± 121, and 3-O-methyldopamine 178 ± 40.3 μg/24 h) were significantly (p < 0.005) higher than in control or amelanotic melanoma subjects. After surgery, there was a substantial decrease in urinary output of L-dopa and its metabolites by these patients.

Additional Keyphrases: urinary excretion of dopa and its metabolites • cancers of ectodermal origin • tyrosine metabolism • monitoring therapy

Melanoma is one of a group of neoplasms, derived histogenetically from the neural crest (1, 2), that includes the retinoblastomas, neuroblastomas, pheochromocytomas, and ganglioneuromas. All of these tumors share an ectodermal cellular origin and are characterized biochemically by abnormal tyrosine metabolism.

In normal melanocytes, L-tyrosine is converted to 3,4-dihydroxyphenylalanine (L-dopa), which is then oxidized to dopa quinone by tyrosinase (EC 1.10.3.1), an enzyme present in the melanin granules. Dopa quinone cyclizes spontaneously to form 2-carboxyindole-5,6-quinone, which on decarboxylation yields indole-5,6-quinone (Figure 1). Further stages of melanin synthesis have not been fully elucidated but appear to include polymerization of indole quinones and (or) carboxy-indole quinones and incorporation of such oligomers into protein structures by formation of sulfur linkages (3–5).

For more than 10 years, increased urinary production of melanin precursors and metabolites (indole, catechol, and phenol melanogens) has been recognized as a specific metabolic disorder in melanoma (5, 6).

Because determination of L-dopa and its metabolites in urine of melanoma patients possibly may specifically disclose a disturbance in tyrosine metabolism by melanoma tumors, we describe here a specific and sensitive enzyme-radioimmunoassay for the quantitative estimation of L-dopa and its metabolites, dopamine and 3-O-methyldopamine. The method is based on the enzymic decarboxylation of L-dopa to dopamine followed by the simultaneous conversion of dopamine in the sample to 3-O-methyldopamine by catechol-O-methyltransferase. The methylated metabolite formed is then determined by radioimmunoassay for 3-O-methyldopamine (7, 8).

With this technique we could measure the 24-h urinary excretion of L-dopa and its metabolites in normal controls, melanoma patients, and other patients in a general-hospital population. We also examined the effect of local excision on the urinary output of these metabolic intermediates in melanoma patients.

Materials and Methods

Apparatus

Liquid scintillation spectrometer (Beckman LS-330; Beckman Instruments, Inc., Spinco Div., Palo Alto, CA 94304).

Centrifuges (Sorvall RC-5B Refrigerated Centrifuge; DuPont Instruments, Pick's Lane, Newtown, CT 06470; and Beckman L5-65; Spinco Div., Palo Alto, CA 94304).

Homogenizer (Polytron; Brinkmann Instruments, Westbury, NY 11590).

Micromedic pipettor (Micromedic Systems, Inc., Horsham, PA 19044).

Evaporator (N-Evap; Organomation Associates Inc., Northborough, MA 01532).

Shaker (Eberbach, Ann Arbor, MI 48106).

Reagents

The cation-exchange resin (Dowex-50W, 50 × 8, 400-mesh, H+ form) was from Sigma Chemical Co., St. Louis, MO 63178. Scintillation fluid (ACS) was from Amersham/Searle Corp., Arlington Heights, IL 60005. All other compounds and solvents were obtained as previously described (7, 8).

Collection of Specimens, and Patient Population

For measurement of L-dopa, dopamine, and 3-O-methyldopamine in urine, 24-h specimens of urine were collected in polyethylene bottles containing 30 mL of 6 mol/L HCl (0°C) and stored at −80°C until analysis.

The analyses were made in the following groups of subjects: (a) healthy normal volunteers, (b) hospitalized patients with no evidence of melanoma, (c) patients with different degrees of melanoma, and (d) a selected group of melanoma patients before and after surgery. The melanoma patients studied were those that were admitted to the Robert Winship Memorial Clinic for Neoplastic Diseases or the Oncology Ward of Emory University Hospital. The diagnosis of melanoma in these patients was verified by histological examination of the tumor and physical examination. The degree of metastasis was...
confirmed by roentgenogram, radioisotope scan, and by computed axial tomography analysis.

The melanoma patients consisted of 14 women and 13 men, age 18 to 76 years. The melanomas were divided into three major clinical groups according to Clark et al. (9): Stage I: Melanoma present as a local disease, either intact, recently biopsied, or locally recurrent after inadequate treatment. Stage II: Melanoma present as a local disease or recently excised for biopsy purposes with evidence of metastases to regional lymph nodes. This also includes melanoma with an unknown primary site. Stage III: Melanoma which has metastasized to distant organs, to multiple lymph node areas, or melanoma that has spread throughout the skin and subcutaneous tissue at the site of original disease.

Under identical conditions, 24-h urine samples were also collected from a group of 10 healthy volunteers of both sexes, 25 to 40 years old, and a group of 10 hospitalized patients with hypertension of various etiology (but excluding pheochromocytoma and neuroblastoma), cirrhosis of the liver, or breast carcinoma.

**Procedures**

**Isolation of L-dopa from urine.** L-Dopa was isolated from urine by ion-exchange chromatography according to a modified procedure of Fahn et al. (10). The cation-exchange resin was purified batchwise by washing successively with 2 mol/L HCl, H2O, 2 mol/L NaOH, H2O, 2 mol/L HCl, and H2O. Chromatography columns (180 × 5 mm) were used; each column contained 1 mL (wet bed) of cation-exchange resin. The pH of the urine sample (20 mL) was adjusted to 2 before it was applied to the column. The column was then washed with 10 mL of water, and the L-dopa in the urine was eluted with 10 mL of sodium phosphate buffer (0.1 mol/L sodium phosphate containing 0.1 mol of NaCl per liter, pH 6.5). L-Dopa in the eluate from the column was diluted fivefold with water and then analyzed by enzyme-radioimmunoassay as described below.

**Enzyme preparation.** An enzymatic preparation containing catechol methyltransferase (EC 2.1.1.6; COMT) enriched with decarboxylase activity was formulated according to the following procedure. Ten Wistar rats were killed by suffocation in a chamber containing solid CO2. Livers (89 g) and kidneys (20 g) were immediately excised and chilled (0 °C). The combined tissues were homogenized with isotonic KCl (400 mL) and centrifuged at 70 000 × g for 40 min. The supernate was decanted, titrated to pH 5 with 1 mol/L acetic acid, allowed to stand for 10 min, then centrifuged at 500 × g for 15 min. The precipitate was discarded and an equal volume of 500 g/L ammonium sulfate solution was added. After centrifugation (500 × g) and dialysis for 6 h vs potassium phosphate buffer (10 mmol/L, pH 7.0), and containing 0.1 mmol of dithiothreitol per liter), 50 mg of dithiothreitol was added to 50 mL of the dialyzed enzyme solution and the mixture was then divided into 1-mL fractions and stored at −80 °C in plastic tubes. The final protein concentration of the enzyme solution was 27 to 35 g/L. The enzyme preparation was stable for at least six months.

**Enzymic assay.** The base for the reaction mixture was composed of Tris·HCl buffer (0.4 mL, 80 mmol/L, pH 8.2), de-ionized glass-distilled water (0.1 mL), MgCl2 (0.2 mL, 24 mmol/L), and S-adenosyl-L-methionine (0.2 mL, 19.2 mmol/L in a mixture of sulfuric acid and ethanol, 9/1 by vol, pH 3). To this would be added one of the following. (a) For the development of a standard curve for dopamine: 0.2 mL, 16.3–163 nmol of dopamine per liter, pH 4.5. (b) For the development of a standard curve for L-dopa: 0.2 mL, 12.7–127 nmol of L-dopa per liter, pH 4.5. (c) For dopamine urinalysis: 0.2 mL of a 30- to 100-fold dilution of the sample (pH 4.5). (d) For L-dopa urinalysis: 0.2 mL of a fivefold dilution (pH 5) of the L-dopa isolated from 20 mL of urine (see section on L-dopa isolation). In each case, the mixture was vortex-mixed and 50 μL of COMT-decarboxylase preparation was added. To determine the presence of 3-O-methyladipamine in the sample alone, we prepared tubes in which 0.25 mL of distilled water was substituted for S-adenosyl-L-methionine (SAM) and COMT. The reaction mixture was incubated for 45 min at 37 °C in a shaking water bath and the reaction was then stopped by adding borate buffer (4 mL, 0.5 mol/L sodium borate, adjusted to pH 10.5 with a 10 mol/L solution of NaOH). We then added 1.5 g of NaCl and 0.3 g of anhydrous Na2CO3. The 3-O-methyladipamine generated in the reaction tubes was then extracted into 30 mL of ethyl acetate by shaking the samples with the organic solvent for 30 min, then centrifuging (500 × g) for 10 min. An 25-μL aliquot of the ethyl acetate was removed. To the remaining two-phase mixture, 10 mL of ethyl acetate was added and, after shaking and centrifuging as above, 10 mL of solvent was withdrawn. The combined fractions of ethyl acetate (35 mL) were evaporated under nitrogen at 40 °C. The residue was reconstituted in 0.4 mL of sodium phosphate buffer (10 mmol/L, pH 7.4) and a 0.3-mL aliquot was analyzed for 3-O-methyladipamine by radioimmunoassay according to a modified procedure of Faraj et al. (7).

**Radioimmunoassay of 3-O-methyladipamine.** In 12 × 75 mm plastic tubes were placed 0.3 mL of bovine serum albumin solution (5 g/L in 10 mmol/L sodium phosphate buffer solution, pH 7.4, containing, per liter, 0.3 g of potassium phosphate, 8 g of NaCl, and 0.5 g of human gamma-globulin, 0.1 mL of antibody solution (2500-fold dilution with phosphate buffer), 0.1 mL of [3H]3-O-methyladipamine (8000 cpm, sp. act. 17 kCi/mol), and either unlabeled 3-O-methyladipamine (0.1 mL, 3–90 nmol/L, pH 7.4, for standard curve) or 0.1 mL of the sodium phosphate buffer (pH 7.4) containing the formed 3-O-methyladipamine extracted from urine after enzymic incubation. The tubes were capped and incubated at 4 °C for 3 h. Antibody-bound 3-O-methyladipamine was separated by adding 0.5 mL of saturated ammonium sulfate according to a modified procedure of Farr (11). The contents of the tubes were vigorously vortex mixed then the tubes were centrifuged at 2000 × g for 30 min at 4 °C. The pellet was washed with 0.5 mL of a 250 g/L ammonium sulfate solution and re-centrifuged. The pellet was then dissolved in 0.5 mL of distilled water and transferred to a counting vial. The tube was washed twice with 3 mL of liquid scintillation fluid. An-
Typical standard curve (log) showing unity between the amount of L-dopa added (6-127 nmol/L) and the 3-O-methyldopamine formed as determined by enzyme-radioimmunoassay. Each point represents an average of 20 determinations (mean and range are shown).

**Fig. 2.**

Analytical recovery. Analytical recovery was assayed by adding known amounts of L-dopa (1.3, 2.6, and 5.2 µmol/L), dopamine (0.33, 0.66, and 1.65 µmol/L), and 3-O-methyldopamine (0.06, 0.3, and 0.6 µmol/L) to previously analyzed urine samples, which were then re-analyzed. The mean analytical recovery of L-dopa, dopamine, and 3-O-methyldopamine for 20 specimens was 65.1 (SD 7.5%), 43.5 (SD 8.3%), and 95.1% (SD 10%), respectively.

Precision. Within- and between-assay variation was assessed for L-dopa, dopamine and 3-O-methyldopamine by assay of 24-h urine samples in the same as well as in a different assay (n = 20), according to the method of Rodbard (12). Intra- and interassay CVs were 14.7 (mean 1.15, SD 0.17), 15.9 (mean 160, SD 25.4), 11.1 (mean 25.1, SD 2.77), 14.7 (mean 1.80, SD 0.26), 14.5 (mean 149, SD 21.6), and 8.9% (mean 31.7 µg/24 h, SD 2.82), respectively, for L-dopa, dopamine, and 3-O-methyldopamine in urine.

**Fig. 3.**

Studies in Humans

In the first part of the study, the concentrations of L-dopa, dopamine, and 3-O-methyldopamine in 24-h urine samples were determined in normal subjects, hospitalized patients without melanoma, and melanoma patients. The results of this study showed that in hospitalized patients with no evidence of melanoma the urinary output of these metabolites did not differ significantly (p > 0.05) from that (av ± SD) of normal persons (L-dopa 1.34 ± 0.3, dopamine 147 ± 38, and 3-O-methyldopamine 31.4 ± 13.6 µg/24 h, Table 1), but were significantly greater in melanoma patients (L-dopa 5.6 ± 1.2, dopamine 555 ± 121, and 3-O-methyldopamine 178 ± 40.3 µg/24 h). Among the melanoma patients, only 30% of those patients with stage I and II, and 80% of those with stage III melanotic melanoma had an abnormal urinary excretion of L-dopa, dopamine, and 3-O-methyldopamine (Table 1). However, in amelanotic subjects, the average urinary output of these metabolites (L-dopa 2.25 ± 0.87, dopamine 154 ± 45.21, and 3-O-methyldopamine 36.5 ± 14.8 µg/24 h) did not differ significantly from that in normal or hospitalized non-melanoma subjects (Table 1).

In the second part of our study, we evaluated the effect of surgery on the metabolism of L-dopa in melanoma patients. The results (Table 2) demonstrated that four of five cases excreted, pre-operatively, abnormal amounts of dopamine, L-dopa, and 3-O-methyldopamine in their urine. After surgery, there was a substantial decrease in the urinary excretion of these metabolites as measured 72 h postoperatively in four of the five patients.

**Discussion**

Here we have described a specific and sensitive assay that allows simultaneous measurement of L-dopa and its metabolites, dopamine and 3-O-methyldopamine, in urine. Aromatic-L-amino-acid decarboxylase (EC 4.1.1.28) and catechol methyltransferase, important enzymes in the biosynthesis and metabolism of catecholamines in the central and peripheral nervous system, were isolated from rat kidney and liver and partly purified by repeated precipitation with ammonium
Table 1. Urinary Excretion of L-Dopa, Dopamine, and 3-O-Methyldopamine by Melanoma Patients

<table>
<thead>
<tr>
<th>Category</th>
<th>No. subjects</th>
<th>L-Dopa (µg/24 h)</th>
<th>Dopamine (µg/24 h)</th>
<th>3-O-Methyldopamine (µg/24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>10</td>
<td>1.30 (0.30)</td>
<td>147.00 (38.0)</td>
<td>31.4 (13.6)</td>
</tr>
<tr>
<td>Patients b</td>
<td>10</td>
<td>1.50 (0.27)</td>
<td>157.00 (41.0)</td>
<td>35.0 (14.2)</td>
</tr>
<tr>
<td>Melanoma</td>
<td>28</td>
<td>5.60 (1.20)</td>
<td>555.00 (121.0)</td>
<td>178.0 (40.3)</td>
</tr>
<tr>
<td>Amelanotic</td>
<td>4</td>
<td>2.25 (0.87)</td>
<td>154.00 (47.2)</td>
<td>36.5 (14.8)</td>
</tr>
<tr>
<td>Melanotic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I and II</td>
<td>16</td>
<td>13.40 (4.60)</td>
<td>338.00 (75.3)</td>
<td>69.43 (27.9)</td>
</tr>
<tr>
<td>Stage III</td>
<td>8</td>
<td>126.60 (27.90)</td>
<td>984.62 (188.4)</td>
<td>125.40 (39.3)</td>
</tr>
</tbody>
</table>

* Average (SD).  b Hospitalized patients with no evidence of melanoma.

Table 2. Urinary Excretion of L-Dopa, Dopamine, and 3-O-Methyldopamine by Melanoma Patients before and after Surgery

<table>
<thead>
<tr>
<th>Subject</th>
<th>L-Dopa</th>
<th>Dopamine</th>
<th>3-O-Methyldopamine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/24 h</td>
<td>µg/24 h</td>
<td>µg/24 h</td>
</tr>
<tr>
<td>Before surgery</td>
<td>After surgery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>493</td>
<td>31</td>
</tr>
<tr>
<td>2</td>
<td>70</td>
<td>860</td>
<td>203</td>
</tr>
<tr>
<td>3</td>
<td>35</td>
<td>400</td>
<td>244</td>
</tr>
<tr>
<td>4</td>
<td>41</td>
<td>826</td>
<td>69.13</td>
</tr>
<tr>
<td>Average (SD)</td>
<td>41.5 (18.1)</td>
<td>595 (173)</td>
<td>137 (89)</td>
</tr>
<tr>
<td>Range</td>
<td>20–70</td>
<td>400–860</td>
<td>31–244</td>
</tr>
<tr>
<td>5</td>
<td>0.8</td>
<td>100</td>
<td>39</td>
</tr>
</tbody>
</table>

sulfate. The determination of catechol methyltransferase activity was based on the principle that the enzyme catalyzes the transfer of methyl groups to substrates containing the catechol moiety in the presence of S-adenosylmethionine as the methyl donor and magnesium ions as the activator. In this case, L-dopa and dopamine were used as the substrates for the enzymatic step (i.e., L-dopa → dopamine). The end product of the enzymatic reaction, 3-O-methyldopamine, was measured by radioimmunoassay (7). In spite of the broad substrate specificity of catechol methyltransferase, all known substrates are characterized by a catechol configuration, regardless of other substituents in the aromatic ring. The unique feature of the enzyme-radioimmunoassay of L-dopa and dopamine is its ability to identify the O-methylated dopamine product directly in the reaction mixture by our specific radioimmunoassay without prior chromatographic separation of the 3-O-methyldopamine from the rest of the methylated catechol derivatives.

A comparison of our method with those reported in the literature demonstrated that our assay for the simultaneous measurement of L-dopa and its metabolites dopamine and 3-O-methyldopamine is precise, sensitive, reproducible, and inexpensive. The ease and simplicity of the assay procedure allows the processing of multiple urine samples per day. The conventional assay of L-dopa and its metabolites in biological fluids has depended on time-consuming extraction procedures coupled with fluorometric analysis (13, 14). Several workers (15, 16) have investigated gas-liquid chromatographic methods for the quantification of these catechols. Although these procedures are promising, problems inherent in preparing biological samples for gas-liquid chromatography and interfering side reactions in the derivatization of L-dopa and its metabolites tend to make this alternative less attractive for routine clinical laboratory analysis for catecholamines.

Our assay of L-dopa, dopamine, and 3-O-methyldopamine was used to determine the 24-h excretion of these metabolites in urine of melanoma patients. The resulting data revealed a preponderance of L-dopa and dopamine in the urine, which increased in parallel with the spread of the disease. Only 30% of the patients with stage I and II exhibited even moderately increased amounts of these catechols. Although the amounts varied widely, even among patients at the same clinical stage of the disease, a general trend toward higher values was recognizable in the more advanced cases. Furthermore, in patients in whom the advanced malignant melanoma (stage III) was also associated with metastases to the liver there was an abnormally high but more common urinary excretion of dopamine.

In view of the observation that the degree of melanogenesis may correlate with the degree of malignancy of the melanoma (6, 17), one may think that the increased urinary output of L-dopa and dopamine by patients with melanotic melanoma is specifically related to the mass of the tumor cells. In support of this thesis, Banda et al. (18), Rosengren et al. (19) and Morgan et al. (20) established the presence of L-dopa and other catechols in extracts of melanotic melanomas. Furthermore, four of five melanoma patients excreting abnormal amounts of melanin metabolites whom we studied showed an appreciable decrease in urinary catechols after surgery. However, patients with amelanotic melanoma whose excretion of these metabolites was normal did not respond biochemically to the surgical ablation of the melanoma tumor.

What are the biochemical mechanisms of the increased urinary production of L-dopa, dopamine, and 3-O-methyldopamine in malignant melanoma? The pathway involved in the formation of melanin is relatively well understood (Figure 1). Although the details of melanin synthesis are yet to be
worked out, the rate-limiting steps in melanogenesis seem to occur at the level of oxidation of tyrosine to L-dopa and L-dopa to dopaquinone. The enzyme tyrosinase catalyzes both conversions. The quantity of melanin synthesized depends on the amount of tyrosinase activity present in the cell, which is regulated by melanotropin (melanocyte-stimulating hormone) (21, 22). Various investigators have attempted to correlate serum tyrosinase assay activity with melanoma. Nishioka et al. (23) measured the activity of this enzyme and found significant increases in patients with malignant melanoma. Increased tyrosinase activity was found to be specific to melanoma patients, because it could not be detected in human cell lines from carcinoma of the breast and colon.

Therefore, increased tyrosinase activity could lead to an accelerated conversion of tyrosine to L-dopa and the formation of a series of metabolic intermediates such as dopamine and 3-O-methyldopamine, which accumulate in the blood or are excreted in the urine in abnormal amounts by patients with melanotic melanoma.

Our findings confirm what has previously been reported regarding the role of L-dopa metabolism in melanoma. Trapeniznikov et al. (24) and Morgan et al. (20) demonstrated that in patients with metastases to regional lymph nodes as well as those with locally disseminated forms of melanotic melanoma of the skin, the concentration of homovanillic acid in urine is increased significantly. Hinterberger et al. (25), Voorhess (26), and Türler and Käser (27) found increased urinary L-dopa and dopamine in patients with melanoma and that the values declined toward normal after treatment. Recently, Blosi and Banda (28) reported their preliminary results on attempts to detect occult metastatic melanoma by ion-exchange column chromatography of urine. They demonstrated increases in five peaks in chromatograms of urine of patients with metastatic disease. The peaks included L-dopa, 3-O-methyldopa, and 5-S-cysteynilpoda. Sustained amplitude of one or more peaks was considered evidence of recurrent or continuing tumor.

In summary, the approach presented here for determination of L-dopa and its metabolites, dopamine and 3-O-methyldopamine, is rapid and reliable. The assay may provide a more specific and comprehensive biochemical profile for detection of melanotic melanoma. It may also be helpful in monitoring the course of this disease and in assessing the efficacy of treatment.

References