Radioenzymatic Assay of DOPA (3,4-Dihydroxyphenylalanine)

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We modified the single-isotope radioenzymatic assay for catecholamines [Life Sci. 21, 625 (1977)] to assay 3,4-dihydroxyphenylalanine (DOPA). DOPA decarboxylase is used to convert DOPA to dopamine, which concurrently is converted to [3H]-3-O-methyl dopamine in the presence of catechol-O-methyltransferase and [methyl-3H]-S-adenosylmethionine and assayed radioenzymatically. For assay of plasma DOPA, 50 μl of untreated plasma is added directly into the incubation mixture. A duplicate mixture containing an internal standard requires a second 50-μl aliquot of plasma. Because the assay measures both DOPA and endogenous dopamine, two additional aliquots of plasma must be assayed for dopamine in the absence of the decarboxylase by the differential assay; DOPA is estimated by difference. The assay is sensitive to 25 pg (500 ng/liter of plasma). Analysis of DOPA (DOPA plus dopamine) and the concurrent differential assay of catecholamines in at least 10 samples can be done in a single working day. Plasma DOPA concentrations for 42 normotensive adults were 1430 ± 19 ng/liter (mean ± SEM). In contrast, dopamine concentrations for these same subjects averaged 23 ± 20 ng/liter. Values for the 24 women subjects (1510 ± 62 ng/liter) significantly (P = 0.04) exceeded those for the men (1320 ± 75 ng/liter).

Additional Keyphrases: sex-related differences · normal values · dopamine · catecholamines · parkinsonism · catecholamine biosynthesis · DOPA in normal plasma

Development of single-isotope radioenzymatic assay methodology for analysis for dopamine (DA), norepinephrine (NE), and epinephrine (E) is making more possible intensive studies of the possible roles of these catecholamines in physiologic and neurologic processes (1-8). In this methodology the enzyme catechol-O-methyltransferase (EC 2.1.1.6) is used to catalyze the transfer of a radioactive methyl group from [methyl-3H]-S-adenosyl-L-methionine to the catecholamine acceptor molecule to form a radioactive O-methyl catecholamine derivative. The sensitivity of this assay methodology, which is in the range of 10–20 fmol, is such that it permits accurate concurrent determination of each catecholamine in very small volumes of blood, cerebrospinal fluid, or other biological fluid.

The precursor role of DOPA in the biosynthesis of the catecholamines is well known (9). The clinical utility of DOPA in alleviating the akinesia and rigidity of Parkinson's disease in specific areas of the brain sufficient to maintain dopaminergic nerve activity in the brain (10, 11) is based rationally upon that precursor function. Recently, several reports have indicated an interest in the mechanisms by which DA and DOPA increase renal blood flow, Na+ and K+ excretion, and glomerular filtration rate (12-15). However, the lack of a sensitive assay for DOPA continues to relegate its role to that of an inconsequential precursor of DA in the synthesis of the catecholamines.

This report describes a rapid assay of DOPA, which is sensitive to 25 pg, requires a maximum of 200 μl of plasma or other fluid, and involves a modification of the radioenzymatic assay methodology for catecholamine analysis (1). We also describe our use of the assay to obtain some data on normal DOPA concentrations in plasma.

Materials and Methods

Standards, reagents, samples, enzyme isolation. L-DOPA, 3-O-methylDOPA, and DL-threo-3,4-dihydroxyphenylserine were purchased from Regis Chemical Co., Morton Grove, Ill. 60053. All other compounds and solvents were obtained as previously described (1).

Plasma samples were obtained with the cooperation of the Bronson Clinical Investigation Unit, Bronson Methodist Hospital, Kalamazoo, Mich. Our subjects, which previously had been found during a physical examination to have a normal blood pressure, entered the clinic and reclined fully supine in a bed in quiet, nonstimulating surroundings for 10–15 min. A large-bore butterfly infusion set was then used to collect blood from the cubital or other convenient arm vein. Blood was sampled 45 min later, i.e., after 60 min of recumbency, into Vacutainer Tubes containing glutathione and ethyleneglycol-bis(β-aminoethyl ether)-N,N'-tetraacetic acid (1). Plasma was obtained by centrifugation; the plasma was removed and placed into a no. 2063 Falcon tube and frozen at −70 °C. DOPA concentrations in control plasma samples prepared and stored in this manner have been stable for one year. Where indicated, other plasma samples were drawn after various conditions as those described above. These samples were shipped on solid CO2 to this laboratory and were then stored, without thawing, at −70 °C. None of the subjects was on any drug treatment.

Enzyme isolation. Catechol-O-methyltransferase activity was purified from rat liver and characterized as previously described (1). After we assessed its activity, this enzyme was diluted with a mixture of tris(hydroxymethyl)methylamine/dithiothreitol (pH 7.4, 1 and 0.1 mmol/liter, respectively) to provide maximal enzyme activity with 10 μl of the preparation/assay tube. A DOPA decarboxylase (EC 4.1.1.28) inhibitor was not added to this preparation.

DOPA decarboxylase activity was partly purified from cardiovascular diseases research, The Upjohn Co., and Upjohn Diagnostics, a division of The Upjohn Co., Kalamazoo, Mich. 49001.

1 Nonstandard abbreviations used: DA, dopamine; NE, norepinephrine; E, epinephrine, and DOPA, 3,4-dihydroxyphenylalanine.

Received June 21, 1978; accepted Aug. 11, 1978.

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2 This enzyme is more appropriately called aromatic-L-amino-acid decarboxylase (EC 4.1.1.28). Because it is stereospecific in its activity, L-DOPA was the isomer used as substrate throughout. Unless specified to the contrary, all other substrates also were the levorotatory isomer.
guinea pig kidneys through the ammonium sulfate fractionation steps of the procedure described by Clark et al. (16). The fraction precipitated by 35–55% saturated ammonium sulfate was dialyzed for 6 h vs. two 4-liter changes of doubly-distilled de-ionized water. Insoluble material in this fraction was then removed by centrifugation at 12,000 X g for 10 min in a Sorvall refrigerated centrifuge and 1-ml aliquots of the supernate were placed in plastic vials, and stored frozen. The protein content of this preparation was 38 g/liter (17). Subsequently, this stock enzyme was diluted 10-fold with water as needed to provide the working enzyme preparation.

Assay. The incubation mixture used in assay of DOPA (plus DA) contains, per liter final concentration, 100 mmol of tris(hydroxymethyl)methylamine, 30 mmol of MgCl2, 10 mmol of ethyleneglycol-bis(β-aminoethyl ether)-N,N'-tetraacetic acid, 10 µl of the transferase preparation, 0.5 µl of the working DOPA decarboxylase preparation, 1 mmol of reduced glutathione, and 4.3–5.5 µmol (5 µCl ≈ 10 µl) of [methyl-3H]-S-adenosyl-L-methionine (New England Nuclear Corp.; spec. acty., 8.8–11.5 kCl/mol). The total incubation mixture, 100 µl in volume, also included 50 µl of plasma. To a duplicate incubation mixture containing a second 50-µl aliquot of plasma sample was added 1 ng of L-DOPA as an internal standard. Only the plasma and standard were omitted from the blank. This use of the two enzymes facilitates the decarboxylation of DOPA to DA followed by O-methylation of the resulting DA to [3H]-3-O-methyl dopamine ([3H]-methoxytyramine). Because 3-O-methylDOPA is a substrate for the DOPA decarboxylase, the order of the enzyme reactions upon a substrate molecule is not a concern. With the above assay mixture, DOPA is assayed in addition to endogenous DA; thus a second pair of incubation mixtures was used, with the differential assay (1), to assay DA—as well as NE and E. These incubation mixtures differed from that described above in the omission of both the DOPA decarboxylase preparation and the above catechol-O-methyltransferase preparation and in their place a 10-µl aliquot of a catechol-O-methyltransferase preparation containing the DOPA decarboxylase inhibitor, benzoylxyamine, was included. The duplicate tube in this series contained 100 pg each of DA (as well as NE and E) as internal standards.

The samples were incubated at 37 °C for 60 min. Isolation of the resulting [3H]-3-O-methylcatecholamine derivatives has been described in detail (1). [3H]-O-methyl derivatives for both assays can be simultaneously extracted and isolated. Results from the DOPA assay, which measures DOPA plus DA, were then corrected by subtracting the DA values obtained by the differential assay.

Statistical analysis of these results was done when indicated by using the one-way t-test of log-transformed data (18).

Results

We assessed the time and enzyme dependence of the decarboxylase-catalyzed assay of DOPA by using DOPA decarboxylase-fortified incubation mixtures, each of which contained 50 µl of horse plasma and a 1-ng internal standard of DOPA. Figure 1 shows the time dependence of the DOPA assay and also the change in enzyme blank activity with duration of incubation. Because the net activity was linearly related to time for 1 h, a 1-h incubation was used in all further studies. This incubation time also coincided with that used for the transferase-catalyzed O-methylation of the catecholamines (1). Figure 2 illustrates the enzyme dependence of the assay by use of aliquots of the DOPA decarboxylase preparation. Activity was maximal with a 0.5-µl aliquot, and we used this volume thereafter. Blank radioactivity markedly increased with the increase in DOPA decarboxylase volume, although this blank activity did decrease slightly during storage.

We assessed linearity of the DOPA assay under the optimal conditions described (Figures 1 and 2); these results are summarized in Table 1. Assay of DOPA standards resulted in a linear slope over the range of 30 to 10,000 pg and the activity averaged 19 cpm/pg per this extended range. Although the radioactivity of the 10 pg standard was too small to be reliably measured, it was consistently above that of the plasma sample. The sensitivity of the assay—i.e., the picogram equivalents in the blank (3)—is 25 pg.

Results in Table 1 also indicate the typical within-run variation in this assay. The average coefficient of variation for the nine groups of three to six determinations each was 6.7%; only the variation within the three values in the 3-ng group greatly exceed that mean.

Between-run variation is indicated in Table 2. In six runs, 12 assays of the control plasma sample for this study gave a coefficient of variation of 8.8% (1581 ± 139 pg/liter, mean ± SD). [Assay results of the same control plasma sample in the

Fig. 1. Time dependence of DOPA assay
A. Net activity for 1 ng of DOPA standard added to a horse plasma sample. Each point is the average of two determinations. B. Radioactivity in the "blank" samples with increasing incubation time. Each point is the average of two determinations. The "blank" contained enzyme and [3H]-S-adenosyl-L-methionine but substrate was omitted.

Fig. 2. Enzyme dependence of DOPA assay
Net activity indicates cpm produced by an internal standard of 1 ng of DOPA in an incubation mixture containing 50 µl of horse plasma. The net activity (indicated as thousands) indicates cpm/pg of DOPA. Duration of incubation: 60 min. DDC, DOPA decarboxylase.
Clinical Research Laboratory of The Upjohn Company for two consecutive months were 1551 ± 161 ng/liter (n = 9; CV, 10.5%) and 1588 ± 142 ng/liter (n = 9; CV, 9.0%), R. T. Smith, personal communication.

The marked specificity of the differential assay for catecholamines including DA has been described (1). The selective extraction and repartitioning steps in this assay methodology and the rapid thin-layer chromatographic resolution of separation in the [3H]-3-O-methyl dopamine, which contribute to that specificity for the determination of DA, have been maintained. However, the addition of the nonspecific decarboxylase to the incubation mixture reduces that specificity. Results summarized in Table 2 show that α-methylDOPA (Aldomet, Merck Sharp & Dohme) interferes in this DOPA assay, and also that the radioactivity produced in this assay by 1 ng of α-methylDOPA is additive with that of the DOPA standard. This indicates that α-methyl-DA, the product of the decarboxylation of α-methylDOPA, which is then converted by catechol-O-methyltransferase to α-methyl-methoxytyramine, has the same mobility on the thin-layer chromatogram as does the methoxytyramine arising from DA. The artificial substrate three-dihydroxyphenylserine (19), which is converted by DOPA decarboxylase directly to NE, did not alter the DOPA values (as measured DA) but did increase the NE values. A metabolite of DOPA, 3-O-methylDOPA (20), did not interfere in this assay.

Our initial results with the assay of plasma samples show that concentrations of DOPA in plasma of humans greatly exceed those of DA (Table 3). As the study progressed, it became apparent that a sex-dependent variation was present. In the 42 subjects in this study the plasma DOPA averaged 1430 ± 49 ng/liter (mean ± SEM); DOPA values in the 24 women subjects were 1510 ± 62 ng/liter, significantly greater than those in men, 1320 ± 75 ng/liter. In these 42 subjects the plasma DA concentrations averaged 23.0 ± 20 ng/liter (range, 0–69).

### Discussion

Present assays for DOPA in plasma and tissue require a sizable sample, followed by an extensive isolation procedure, including adsorption onto and elution from an alumina column, followed by ion-exchange column chromatography (21–23). DOPA in the resulting eluate is then subjected to fluorometric analysis with a maximum sensitivity of 10 ng (23). Because the fluorophore which develops can arise from other catechol molecules, the specificity of the procedure depends on efficient isolation in the chromatographic steps.

#### Table 1. Linearity of DOPA Assay

<table>
<thead>
<tr>
<th></th>
<th>Gross cpm ± SD</th>
<th>Net cpm</th>
<th>CV %</th>
<th>cpm/pg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>470 ± 34</td>
<td>—</td>
<td>7.2</td>
<td>—</td>
</tr>
<tr>
<td>Plasma (50 µl)</td>
<td>677 ± 42</td>
<td>—</td>
<td>6.2</td>
<td>—</td>
</tr>
<tr>
<td>+10 pg DOPA</td>
<td>775 ± 63</td>
<td>98</td>
<td>8.1</td>
<td>10</td>
</tr>
<tr>
<td>+30 pg DOPA</td>
<td>1312 ± 101</td>
<td>645</td>
<td>8.0</td>
<td>21</td>
</tr>
<tr>
<td>+100 pg DOPA</td>
<td>2577 ± 47</td>
<td>1910</td>
<td>1.9</td>
<td>20</td>
</tr>
<tr>
<td>+300 pg DOPA</td>
<td>7272 ± 474</td>
<td>6805</td>
<td>6.5</td>
<td>22</td>
</tr>
<tr>
<td>+1000 pg DOPA</td>
<td>18314 ± 880</td>
<td>17670</td>
<td>4.8</td>
<td>18</td>
</tr>
<tr>
<td>DOPA</td>
<td>55851 ± 6130</td>
<td>55183</td>
<td>11.0</td>
<td>18</td>
</tr>
<tr>
<td>+10000 pg</td>
<td>17204 ± 11376 169560</td>
<td>6.5</td>
<td>17</td>
<td></td>
</tr>
</tbody>
</table>

Average cpm/pg (30 pg–10 000 pg); 19; pg equivalents in blank: 25. Each value is the average ±SD of at least three determinations.

#### Table 2. Interference in the Assay of DOPA

<table>
<thead>
<tr>
<th></th>
<th>Total cpm</th>
<th>Net cpm</th>
<th>cpm/pg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma (50 µl)</td>
<td>1120</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>+1 ng DOPA</td>
<td>19 314</td>
<td>18 194</td>
<td>18.2</td>
</tr>
<tr>
<td>+1 ng α-methylDOPA</td>
<td>9910</td>
<td>8790</td>
<td>8.8</td>
</tr>
<tr>
<td>+10 ng α-methylDOPA</td>
<td>44 248</td>
<td>43 128</td>
<td>4.3</td>
</tr>
<tr>
<td>+1 ng α-methylDOPA</td>
<td>28 645</td>
<td>27 525</td>
<td>13.8</td>
</tr>
<tr>
<td>+1 ng DOPA</td>
<td>938</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>+1 ng three-DOPA b</td>
<td>992</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>Plasma (50 µl)</td>
<td>18 631</td>
<td>17 693</td>
<td>17.7</td>
</tr>
<tr>
<td>+1 ng DOPA</td>
<td>816</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>+3 ng 3-O-methylDOPA</td>
<td>839</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>+1 ng 3-O-methylDOPA</td>
<td>19 548</td>
<td>18 610</td>
<td>18.6</td>
</tr>
<tr>
<td>+1 ng DOPA</td>
<td>18 930</td>
<td>17 992</td>
<td>18.0</td>
</tr>
<tr>
<td>+3 ng 3-O-methylDOPA</td>
<td>18 930</td>
<td>17 992</td>
<td>18.0</td>
</tr>
</tbody>
</table>

* Each value is the average of duplicate determinations.

* a-three-Dihydroxyphenylserine increased the radioactivity in the NE assay by 2851 cpm, equivalent in that assay run to 109 pg of NE.

#### Table 3. Plasma DOPA in Normotensive Supine Adult Subjects

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SEM</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>All subjects</td>
<td>42 1430</td>
<td>49</td>
<td>925–2380</td>
</tr>
<tr>
<td>Women</td>
<td>24 1510</td>
<td>62</td>
<td>925–2380</td>
</tr>
<tr>
<td>Men</td>
<td>18 1320 a</td>
<td>75</td>
<td>934–2000</td>
</tr>
</tbody>
</table>

* All DOPA concentrations exceeding 1000 ng/liter were rounded to the nearest 10 ng/liter. DOPA values for a "control human plasma sample" used as a quality control sample averaged 1581 ± 42 ng/liter (mean ± SD; SD = 139) for 12 analyses on six different days.

* a Differ significantly from value for women (P = 0.04)

Recently, a similar radioenzymatic assay system was described for measuring tissue DOPA. After the O-methylation a cation-exchange chromatographic step was used in this method, followed by charcoal adsorption and elution of the [3H]-3-O-methylDOPA (24). The sensitivity was increased to approximately 100–200 pg by this still-laborious methodology.

In the method described here we used established radioenzymatic methodology (1–8) with demonstrated specificity to provide a rapid assay for DOPA with sensitivity of about 25 pg (~0.1 pmol). The specificity of this methodology for the catecholamines has been indicated previously (1); that specificity has been confirmed in various other methods based on radioenzymatic assay and chromatographic separation of the [3H]-3-O-methyl catecholamine derivatives (2–8). However, the incorporation of the nonspecific DOPA decarboxylase decreases the specificity in those clinical or research situations in which α-methylDOPA may be used.

The sensitivity of this type of radioenzymatic assay is related to the radioactivity that appears in the blank sample; it is proportional to the amount of enzyme utilized and, to a lesser extent, to the duration of incubation.

This report also shows the marked contrast between the concentrations of circulating DOPA and those of DA (Table 3). In this and other similar methods, DA concentrations in the plasma are generally below the level of sensitivity of the assay (1–4, 6, 8). Because plasma DA is at the limit of detection, analyses for plasma DOPA can be accurate even if only two assay tubes, or 100 µl of plasma, are used, and omitting the differential assay. DOPA may be similarly assayed in ce-

**CLINICAL CHEMISTRY, Vol. 24, No. 11, 1978 1929**
rebrosplinal fluid. Such samples should not be collected with the glutathione and ethylene-bis(β-aminomethyl ether)-N,N'-tetraacetic acid additives solution because DOPA decarboxylase activity is sensitive to excess sulphydryl groups. DOPA concentrations in plasma and cerebrospinal fluid are sufficiently high (>1 mg/liter) that only 25 µl of these fluids can be used in each incubation mixture. DOPA concentrations in urine and in the supernates of tissue homogenates can be assayed without additional isolation of DOPA and require a dilution of as much as 50-fold before assay; because DA concentrations are very significant in these fluids, DA plus DOPA must also be determined for an accurate DOPA assay (Johnson, Gren, Smith, and Baker, personal communication).

The concentration of DOPA in plasma was sex-related in this study. The two groups were not matched for age, but we could discern no age-related effect on the DOPA. The methodology is now available to explore further the questions that these results may raise. The contrast in DOPA values appears to correlate with the increased plasma NE values in women and the slower rate of disposition of NE in women after intravenous NE infusion (Hollenberg, Adams, Borucki, and Johnson, manuscript in preparation).

The assay of DOPA in plasma has been primarily interjected into clinical studies aimed at evaluating the efficacy of L-DOPA therapy in subjects afflicted with parkinsonism or other related neurologic disorders. Problems with fluorometric assays, especially their insensitivity, have precluded more widespread research or clinical examinations of this precursor of catecholamine biosynthesis. This initial report of the normally occurring presence of DOPA in plasma, which can be routinely measured with a rapid, specific, and sensitive assay, should stimulate interest in this intermediate and in factors involved in its formation.

References