Fluorometric Method for Quantitatively Estimating Urinary Dihydroxyphenylethylamine (Dopamine), Dihydroxyphenylalanine (Dopa), and Dihydroxyphenylacetic Acid (Dopac)

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A relatively simple, exact method is described for simultaneously estimating dopamine, dopa, and dopac in urine. The compounds are concentrated on alumina by an abbreviated batch technique and separated by "two solutions" paper electrophoresis at low voltage for 2 h. Fluorophores are developed by condensation with ethylenediamine. The fluorescent bands, eluted into water, are measured fluorometrically. This method was used to estimate the major free metabolites of dopa in urines from parkinsonian and hyperkinetic patients, and normal subjects. Compared with normal controls, dopamine excretion tended to be decreased in patients with parkinsonism, increased in hyperkinetic patients. The sum of the free metabolites (dopamine, dopac, and homovanillic acid) was significantly smaller for the patients with parkinsonism than for normal subjects.

Additional Keyphrases parkinsonism • hyperkinesia • paper electrophoresis
• fluorophores with ethylenediamine • diagnostic criteria

Several methods are currently used to estimate dopamine in urine, but dopac excretion has not been investigated in patients with Parkinson's syndrome. Most of the methods include concentration of dopamine on columns of Al₂O₃ (1) followed by specific estimation, either by fractionation on suitable resins (2, 3) before producing fluorophores, or by selecting the characteristic fluorescence afterwards at specific conditions (4–6). The latter requires highly sensitive instrumentation.

Subnormal amounts of dopamine appear in the urine of patients with Parkinson's disease (7, 8), an observation which can be related to the low dopamine concentration found in the basal ganglia of these patients (9). However, supranormal amounts of dopamine appear in the urine of patients with extrapyramidal hyperkinetic syndromes (8).

This significance of dopamine excretion in neurological disorders and the need to follow the clinical course of many cases led us to develop this method for estimating dopamine. By this technique, dopa, dopamine, and dopac can be simultaneously estimated.

Materials and Method

Apparatus

• Tank for electrophoresis, preferably Universal electrophoresis apparatus after Kohn (Shandon Scientific Co., Sewickley, Pa. 15143).
• Ultraviolet light source, shortwave (250 nm).
• Ratio fluorometer (Beckman Instruments, Fullerton, Calif. 92634) with a single mercury-source lamp and a phosphorus-coated sleeve.

Reagents

All reagents were prepared with glass-distilled water, which is also used in the entire procedure.
1. Activated alumina ("Merck," Brinkmann
Instruments, Westbury, N. Y. 11590): 100 g of \( \text{Al}_2\text{O}_3 \) and 500 ml of 2N HCl are heated in the autoclave for 20 min, washed with distilled water until the pH is slightly acid, and dried at 110°C.

2. \( \text{NaOH}: 2 \text{ g/100 ml of water.} \)
3. \( \text{CH}_3\text{COOH}: 10 \text{ ml per 100 ml of water.} \)
4. **Buffers for “two solutions” electrophoresis (10–12):**
   (a) \( \text{CH}_3\text{COOH}, 0.75 \text{ ml/100 ml, for anode compartment.} \)
   (b) \( \text{CH}_3\text{COOH}, 2.5 \text{ ml/100 ml, for cathode compartment.} \)
5. **Staining solutions:**
   (a) Ethylenediamine, analytical grade, redistilled if necessary.
   (b) Sodium carbonate, 10 g/100 ml of water, freshly prepared once a week.
   (c) Human albumin (Cutter Labs., Berkeley, Calif.), 25 g diluted to 100 ml with water.

**Preparation:** Five milliliters of \( a \) and 20 ml of \( b \) are mixed well. After the mixture is cooled to room temperature, 0.2 ml of \( c \) is mixed in. This reagent should be freshly prepared.

6. **Standard solutions:**
   (a) Dopamine: 12 mg/100 ml, made by diluting 14.8 mg of dopamine hydrochloride to 100 ml with diluted ethanol (15 ml plus 85 ml of water).
   (b) Dopa: 12 mg of dopa in 100 ml of diluted ethanol (15 + 85).
   (c) Dopac: 19.2 mg of dopac, cyclohexylammonium salt in diluted alcohol (15 + 85).
   (d, e, f): 0.6 mg of dopamine, dopa, and dopac, respectively, per 100 ml. To 5 ml each of \( a, b, c \) 20 ml of urea (10 g/100 ml) and 50 ml of dilute \( \text{CH}_3\text{COOH (10 ml/100 ml) are added, and the mixture is diluted to 100 ml with distilled water.} \)

**Procedure**

All glassware is acid-washed and rinsed with glass-distilled water.

Freshly voided urine is examined, or urine stored frozen no longer than 48 h. A volume of urine containing 50 mg of creatinine is placed in a preweighed, round-bottomed, wide-mouthed 100-ml centrifuge tube containing 1.25 g of activated alumina and a magnetic stirring bar. With the aid of a magnetic stirrer and a pH meter, the pH of the urine is adjusted to 8.5 by slowly adding reagent 2 (the stirring should be brisk enough to keep the alumina suspended). Stirring is then continued at pH 8.5 for 5 more minutes, adding NaOH solution as necessary to keep the pH at 8.5. The procedure from alkalization to pH 8.5 until the acetic acid is added should not take more than 15 min, as these metabolites deteriorate quickly at this pH.

After the adsorption is completed, the tube is immediately centrifuged for 1 min and the supernatant fluid decanted. The alumina is rinsed twice with 50 ml of water, again centrifuging and decanting the supernatant fluid as before. The last centrifugation is for 2 min and the rinsing water is decanted as completely as possible. Immediately, 1.25 ml of reagent 3 is added and the centrifuge tube reweighed to determine the volume of the eluate. For elution, the suspension is stirred on the magnetic stirrer for 10 min, then centrifuged in a small test tube.

The whole eluate corresponds to 50 mg of creatinine, the amount in the urine treated with the alumina. For electrophoresis in the “two solutions” system (11), a volume of the eluate equivalent to 1 mg of creatinine is applied in duplicate as a 2-cm streak, 7 cm from the anode end of the filter paper (Schleicher & Schüll, Keene, N. H. 03431; 2043b mg/l, 25 X 5 cm) and is dried in a stream of cool air. Both ends of the filter paper strips are wetted with the respective acetic acid solutions until the liquid nearly reaches the application line. For 2 h, 350 V (about 1 mA per strip) is applied. The strips are then dried at 80°C for 10 min and stained with freshly prepared reagent 5, wetting sparingly without immersing. The fluorophor develops completely within 90 min and the strip is then inspected under ultraviolet light (Figure 1A). The paper strips should be handled carefully throughout the procedure and placed only on clean glass for examination. The fluorescent bands are marked with a pencil, cut out, and eluted into 5 ml of water. Bands of identical size from the nonfluorescent parts of the same filter paper strip serve as a blank. After it has been slightly shaken and has stood at room temperature at least 3 h (to overnight), or at 50°C for 45 min (13), the supernatant fluid is removed after centrifugation, and read in a fluorometer. The phosphorus-coated sleeve is adjusted at 450 nm; two primary filters UG 11 and a secondary filter selecting fluorescence at 420 nm are used. The uranium glass rod no. 4 serves as reference.

Freshly prepared dopamine, dopa, and dopac solutions are used for calibration. Once the standard curve has been established and compared with

![Image](https://via.placeholder.com/150)

**Fig. 1. Example of electrophoretic separation**

A, normal urine concentrated on \( \text{Al}_2\text{O}_3; \) B, C, unconcentrated urine of patient during treatment with L-dopa
Table 1. Urinary Excretion of Dopa Metabolites (μg/g of Creatinine) in Patients with Different Disorders

<table>
<thead>
<tr>
<th>Free metabolite</th>
<th>Normal</th>
<th>Parkinson's disease</th>
<th>Hyperkinetic syndromes</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Range</td>
<td>Mean ± SD</td>
<td>Range</td>
</tr>
<tr>
<td>Dopamine</td>
<td>(28)* 100-410</td>
<td>267 ± 79</td>
<td>(51) 50-310</td>
</tr>
<tr>
<td>Dopac</td>
<td>(22) 1070-3540</td>
<td>1825 ± 776</td>
<td>(35) 340-1320</td>
</tr>
<tr>
<td>Homovanillic acid</td>
<td>(14) 3300-15000</td>
<td>6893 ± 2830</td>
<td>(26) 500-6000</td>
</tr>
<tr>
<td>Dopamine + dopac</td>
<td>(22) 1280-3842</td>
<td>2071 ± 867</td>
<td>(35) 455-1630</td>
</tr>
<tr>
<td>Dopamine + dopac +</td>
<td>(14) 6335-16760</td>
<td>9092 ± 2731</td>
<td>(26) 1330-6900</td>
</tr>
<tr>
<td>homovanillic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Numbers in parenthesis are number of samples examined.

that obtained by using uranium rods no. 2 and 3, these rods subsequently can serve as supplementary standards.

The standard curves are made by applying, for example, 0.15, 0.3, and 0.6 μg of dopamine, dopa, or dopac (from the 6 d, e, or f solutions) to filter paper strips, which are treated like those of the sample.

For recovery of each of the three metabolites, the whole procedure is performed simultaneously on two samples of the same urine, 60 μg of the respective standard (0.5 ml of 6 a, b, or c solution) being added to one, and thereafter proceeding exactly as has been already described.

Results and Discussion

A recovery of 90 ± 2% (range) was obtained for dopamine, 49 ± 3% for dopa, and 31 ± 3% for dopac, each of these values being based on 10 examinations. A linear relation was found between the quantities of these metabolites and their fluorescence intensity. Equal quantities of the three metabolites emit different amounts of fluorescent quanta, the ratio being 1:2:3 for dopamine, dopa, and dopac, respectively. Since this ratio is inversely proportional to the recovery of the three substances by the method employed, the actual fluorometric readings are almost identical for the three metabolites at equal concentrations.

As for reproducibility, the standard error for duplicate estimations is 5.6% for dopamine and 4.1% for dopac. The reproducibility of the method is greatly enhanced by adding human albumin to the staining solution: the fluorophores are eluted better and blank values are decreased. Evidently the protein coats the cellulose fibers of the filter paper, thus hindering nonspecific fluorescent substances from leaving the paper during elution.

Careful staining with ethylenediamine is necessary to avoid diffusion of the fluorescent bands. Clearcut bands result if the filter paper is passed carefully through a small amount of fluid.

The urines of 51 patients with parkinsonism, 19 patients with various hyperkinetic syndromes, and 28 normal controls were examined for dopamine and, in many cases, also for dopac and HVA1 (Table 1), the latter being estimated according to Armstrong et al. (14).

Dopac and HVA were estimated in addition to dopamine because circulating dopamine is easily metabolized by monoami- nooxidase [monoamine: oxygen oxidoreductase (deaminating), EC 1.4.3.4] to dopac, and further, by catechol methyltransferase (S-adenosylmethionine:catechol O-methyltransferase, EC 2.1.1.6) to the final metabolite, HVA (Figure 3). More complete information on dopa turnover is obtained when all three metabolites are measured and their sum considered. Dopac, however, was not estimated in any of these subjects since it is excreted in such minute quantities (normal value, 50 μg/g of creatinine).

All the patients with Parkinson’s syndrome were examined before L-dopa was administered, but during conservative anti-Parkinson treatment [with “Disipal,” “Artan,” “Cogentine,” and (or) “Ponalide”].

Patients receiving methyl-dopa (“Aldomin”) and MAOI were not included in this study. Methyldopa seriously interferes with the estimation of the metabolites of dopa because it causes an excretion of methyldopa and methyl-dopamine. A parkinsonian patient receiving methyl-dopa gave the following false results: 1420 μg/g of creatinine for dopamine, and 5700 μg/g of creatinine for dopa. The estimation of these metabolites is also interfered with by the administration of MAOI, which causes increased excretion of dopamine and dopa and decreased excretion of dopac.

The urinary excretion of free dopamine, dopac, and HVA in parkinsonian, hyperkinetic, or normal subjects is summarized in Table 1.

The above data are detailed in Figure 2. Even without a statistical test, it is obvious that the mean excretion of dopamine was significantly less

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1 Abbreviations used: HVA, homovanillic acid; MAOI, monoamineoxidase inhibitors.
in the parkinsonian patients than in the normal controls (Figure 2A). The mean excretions of dopac and HVA were even more depressed, both individually (Figure 2B and C) and expressed as the sum of dopamine + dopac, as well as the sum of dopamine + dopac + HVA (Figure 2D and E). Patients with hyperkinetic syndromes excreted significantly greater amounts of dopamine than did parkinsonians or normal controls (Figure 2A).

However, we would like to compare the five criteria used in diagnosing parkinsonism (Table 2). For this purpose, we made the following computations under the assumption that the measurements are normally distributed and taking the sample means and sd's for the population means and its sd's. We computed for each criterion $i$, a critical value $A_i$ at $\alpha = 0.05$. Thus, if for a patient the measurement $X_i$ was $< A_i$ he belonged to the parkinsonian group; and if $X_i$ was $> A_i$ he did not belong to it. $A_i$ is such that, under the above assumption, we would have an error of the first kind (a) equal to 0.05, which means that we would consider 5% of the cases to be false negative. We did compare for the five criteria the values of $\beta_i$, the error of the second kind, namely the proportion of the normal controls which would be diagnosed as parkinsonian under each method. The lowest value of $\beta$ corresponds to the best diagnostic criterion.

Generally, our findings accord with previous ones (5, 7). Thus, in patients with Parkinson's syndrome, the values for dopamine were decreased, while in our patients with extrapyramidal hyperkinetic syndromes they tended to be increased. A high excretion of dopamine has been reported in patients with chorea and segmental dystonia (7).
Table 2. Comparison of Errors of the Second Kind (\(\beta\)) for the Five Criteria at \(\alpha = 0.05\)

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Critical point A</th>
<th>Controls overlapping into parkinsonians, % (\beta)</th>
</tr>
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<tbody>
<tr>
<td>1 dopamine</td>
<td>293</td>
<td>0.63</td>
</tr>
<tr>
<td>2 dopac</td>
<td>1084</td>
<td>0.17</td>
</tr>
<tr>
<td>3 HVA</td>
<td>5700</td>
<td>0.35</td>
</tr>
<tr>
<td>4 dopamine + dopac</td>
<td>1329</td>
<td>0.18</td>
</tr>
<tr>
<td>5 dopamine + dopac + HVA</td>
<td>6656</td>
<td>0.19</td>
</tr>
</tbody>
</table>

Table 3. Urinary Excretion of Dopa and Its Metabolites in Two Cases of Tumors

<table>
<thead>
<tr>
<th></th>
<th>Dopa</th>
<th>Dopamine</th>
<th>Homovanilllic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\mu g/g) of creatinine</td>
<td>(\mu g/g)</td>
<td></td>
</tr>
<tr>
<td>Ganglioneuroma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before operation</td>
<td>low</td>
<td>1,280</td>
<td>5,760</td>
</tr>
<tr>
<td>After operation</td>
<td>not detectable</td>
<td>250</td>
<td>700</td>
</tr>
<tr>
<td>Neuroblastoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before treatment</td>
<td>4,000</td>
<td>42,400</td>
<td>44,000</td>
</tr>
<tr>
<td>After 2-month treatment with “Endoxan”</td>
<td>1,335</td>
<td>6,800</td>
<td>11,400</td>
</tr>
</tbody>
</table>

Fig. 3. The metabolic pathway of dopa

Among our cases, an unusually high excretion of dopamine (about 6 \(\mu g/g\) of creatinine) was found in a patient with thyroid dysfunction and marked involuntary movements (15). (This outstanding value was not included in the statistical evaluation.)

Dopa and its metabolites were also measured in the urine of one patient with ganglioneuroma, before and after surgery, and in one patient with neuroblastoma with bone metastases, before and during treatment with “Endoxan” (Table 3).

This method is also applicable to the urine of parkinsonians treated with L-dopa. Dopa, dopamine, and dopac are detectable even after low doses of L-dopa (1 to 2 g daily) without previous concentration of the urine (Figure 1B and C).

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References