Differential Assay for Urinary Catecholamines by Use of Liquid
Chromatography with Fluorescence Detection

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I describe a relatively simple and rapid procedure for quantitative analysis for unconjugated urinary norepinephrine, epinephrine, and dopamine. Catecholamines are isolated from urine on a carboxylic acid ion-exchange resin (Bio-Rex 70) and eluted with 0.8 mol/L boric acid. The eluates are assayed for the individual catecholamines by "high-performance" liquid chromatography with an octadecylsilane reversed-phase column and 10 mmol/L perchloric acid–acetonitrile (99/1) as the mobile phase. The catecholamines are detected by measuring their intrinsic fluorescence on excitation at 200 nm. The method is highly reproducible, the coefficient of variation for normal concentrations in urine being 4.4% for norepinephrine, 8.8% for epinephrine, and 4.8% for dopamine. Moreover, it appears to be practically free of interference by drugs or endogenous compounds in urine. The range of catecholamine concentrations found in 143 hypertensive patients agrees well with previous studies.

Additional Keyphrases: pheochromocytoma • hypertension • cancer • norepinephrine, epinephrine, dopamine excretion by the hypertensive

For detection of pheochromocytoma, measurement of urinary catecholamine metabolites such as metanephrine or vanilmandelic acid has been preferred in the past to the measurement of the catecholamines, because the metabolites are present in much greater quantity and are thus easier to measure (1).

"High-performance" liquid chromatography (HPLC) has been proposed for use in the assay of urinary catecholamines after the catecholamines have been extracted with alumina under alkaline conditions and eluted with dilute acid. The eluates were then assayed by HPLC with detection by virtue of their ultraviolet absorbance (2) or their fluorescence after reaction with o-phthalaldehyde (3). Recently, Moyer et al. (4) described an HPLC method for assay of urinary catecholamines, but they found it necessary to purify alumina eluates further, by use of a borate gel, before chromatography. In that study, which involved 22 patients with surgically proven pheochromocytoma in addition to normal and hypertensive subjects, it was found that the catecholamine assay had better predictive value for the presence of the tumor than the metanephrine assay, although its technical difficulty made it less suitable for use as a routine screening test.

We recently described a simplified method for the assay of urinary catecholamines (5), with use of disposable columns of the ion-exchange resin Bio-Rex 70 for initial sample purification. The resin eluates are assayed by HPLC according to a procedure previously described for assay of catecholamines in brain (9). The catecholamines are detected by their intrinsic fluorescence on excitation at 200 nm. The procedure has now been applied to samples of urine from hospital in- and outpatients, and appears to be almost free of interference by other endogenous compounds or drugs administered in therapeutic doses, while giving results that are in good agreement with previous studies (2, 4).

Materials and Methods

Materials

[7-3H]-D,L-Norepinephrine (5 to 15 kCi/mol) was obtained from New England Nuclear, Boston, MA, 02118. Norepinephrine, epinephrine, and dopamine hydrochlorides were from Sigma Chemical Co., St. Louis, MO 63178. Cation-exchange resin columns for catecholamine analysis, containing about 1 g of resin, were obtained from Bio-Rad Laboratories, Richmond, CA 94804. Acetonitrile, "190-nm HPLC" grade, was purchased from Waters Associates, Milford, MA, 01757. Instagel was purchased from Packard Instrument Co., Downers Grove, IL, 60515. All other reagents were of "ACS-certified" grade.

Reagent Preparation

Catecholamine stock solutions (1 g/L) were prepared in 10 mmol/L hydrochloric acid and stored at 4 °C for as long as two months. Dilute standards (1 mg/L) were prepared freshly each day in 10 mmol/L hydrochloric acid. Tritiated norepinephrine was diluted 50-fold with 0.1 mol/L tartaric acid and kept at 4 °C for as long as two weeks. Water for use in liquid chromatography was redistilled from alkaline permanganate before use.

Urine Samples

Urine samples were obtained from the Alfred Hospital as part of the normal screening process for hypertensive patients. Twenty-four-hour urine specimens were collected in polyethylene bottles with 15 mL of concentrated hydrochloric acid as preservative and kept at 4 °C. The urine volume was recorded and a 100-mL aliquot was stored at −20 °C until assayed.

Assay Procedure

To a 5-mL aliquot of urine, add 15 mL of disodium EDTA (1 g/L) and titrate the mixture to pH 6.5 ± 0.1 with sodium hydroxide (0.5 mol/L), using a pH meter. Resuspend the resin in a Bio-Rad column for urinary catecholamine analysis, shaking to remove air pockets, and allow to drain. Allow the urine sample to pass through the column, then wash the column twice with 20 mL of water. Add 1 mL of boric acid (0.8 mol/L) to the column and discard the effluent. Add a further 5 mL of boric acid and collect the eluate for assay.

Analytical recovery of norepinephrine was monitored by adding tracer amounts of tritiated norepinephrine (100 nCi) to samples before extraction and determining the fraction recovered in the eluates by liquid scintillation counting. To assess the recoveries of the other amines, I added 2 µg of each catecholamine to 5-mL aliquots of pooled urine and determined the absolute recovery by analyzing the boric acid eluates by liquid chromatography as follows.
Chromatography of Catecholamines

A 5000 series liquid chromatograph with universal loop injector and a Micropak MCH-10 octadecylsilane reversed-phase column, 300 x 4 mm i.d., were obtained from Varian Associates, Palo Alto, CA 94303. A 40-mm guard column was packed with pellicular octadecylsilane material (Vydac SC reverse phase, also from Varian Associates). The detector was a Schoeffel FS 970 fluorometer (Schoeffel Instrument Co., Westwood, NJ 07675) fitted with a deuterium arc source. The excitation monochromator was set at 200 nm; emission wavelengths were selected with a Corning 7-60 glass filter having an approximate passband of 320 to 400 nm.

The mobile phase was a mixture of 0.01 mol/L perchloric acid/acetonitrile (99/1 by vol) at a flow rate of 2 mL/min. Various amounts of the boric acid eluates (50 to 100 µL) were injected onto the column with a precision microsyringe. For each analysis, a calibration curve was constructed by injecting known amounts of each catecholamine (2 to 20 ng). After use, the column was flushed with acetonitrile and stored filled with that solvent.

Results and Discussion

The weak carboxylic acid ion-exchange resin, Bio-Rex 70, has been extensively used for isolation and assay of catecholamines and their metabolites (6–8) and forms part of a commercially available assay kit from which the disposable columns used in this procedure were taken. The mean analytical recovery of 2 µg of each catecholamine when added to 5 mL of pooled urine was determined with use of the chromatographic system described. The mean recovery of norepinephrine (n = 6) was 74.5% (SD 3.8%) and in good agreement with the tritiated norepinephrine recovery, 75.4% (SD 7.4%). The recovery of dopamine was 80.5% (SD 5.2%), a difference of 8%. This small difference would make it possible to use either tritiated norepinephrine or dopamine to monitor recovery. The recovery of epinephrine, 92.5% (SD 3.2%) was 24% higher than that of norepinephrine, so that if more accurate measurement of epinephrine is required, its recovery should be separately determined. The results here have not been corrected for these differences in recovery.

The liquid-chromatographic assay procedure has been previously described (9) for the assay of catecholamines in brain. The standard curve is linear and results are reproducible when amounts ranging from 1 to 50 ng of catecholamine are injected. Typical regression equations for this range are: y = 14.4x - 7.3 for norepinephrine, y = 9.43x - 4.0 for epinephrine, and y = 4.20x + 0.8 for dopamine, where y is the chromatographic peak height in nanoamperes of detector current and x is nanograms of amine. The CV of the detector response to the injection of 2 ng of any catecholamine was less than 10% for any one assay, or for all assays over a two-month period. The between-assay CV (n = 5) for a typical urine sample containing 120 ng of norepinephrine, 60 ng of epinephrine, and 1200 ng of dopamine in 5 mL was 4.4% for norepinephrine, 8.8% for epinephrine, and 4.6% for dopamine. A chromatogram of the eluate from this sample has been illustrated (5). The limit of sensitivity is approximately 100 pg for norepinephrine, 200 pg for epinephrine, and 300 pg for dopamine, corresponding to 2 to 6 µg/24 h excreted in the urine if a urinary volume of 1 L and a 50% recovery are assumed. Total chromatography time is 6 min. Longer-term reproducibility and stability were assessed by adding catecholamines to pooled urine in amounts comparable with those already present. Aliquots of the urine were stored at -20 °C for two months.

![Chart](image_url)

Table 1. Long-term Reproducibility and Stability of Urine Assay *

<table>
<thead>
<tr>
<th>Norepinephrine</th>
<th>Epinephrine</th>
<th>Dopamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Found (CV, n = 6)</td>
<td>Added</td>
<td>Found (CV)</td>
</tr>
<tr>
<td>33.3 (5.5)</td>
<td>0</td>
<td>N.D.</td>
</tr>
<tr>
<td>54.7 (7.1)</td>
<td>20</td>
<td>19.0 (12.6)</td>
</tr>
<tr>
<td>77.5 (12.6)</td>
<td>40</td>
<td>33.0 (18.2)</td>
</tr>
<tr>
<td>116 (7.0)</td>
<td>80</td>
<td>57.0 (15.8)</td>
</tr>
<tr>
<td>191 (8.5)</td>
<td>160</td>
<td>95.5 (7.2)</td>
</tr>
</tbody>
</table>

* Known amounts of catecholamines were added to aliquots of pooled urine and stored for as long as three months before assay. N.D., not detected.
and assayed during three months. The results (Table 1) confirm that the recoveries of norepinephrine and dopamine are similar, while that of epinephrine is higher, although the CV for epinephrine reflects the lower sensitivity for this amine. There appears to be no loss of catecholamine during this time.

Urine samples from 143 hypertensive patients were assayed and the distribution of results is shown in Figure 1. Because the results do not appear to be normally distributed, means and normal ranges were calculated in the same way as those of Moyer et al. (4). They are 36.7 μg/24 h (9–83 μg) for norepinephrine, 7.0 μg/24 h (0–34 μg) for epinephrine, and 249 μg/24 h (72–527 μg) for dopamine. The sum of norepinephrine and epinephrine ranged from 9 to 98 μg, mean 42.7 μg/24 h. If the differences in amine recoveries are taken into account, these results agree well with those of Moyer et al. The mean recovery of tritiated norepinephrine in these samples was 77% (range 40–96%). Of the drugs taken concurrently by these patients, only L-dopa and α-methyltyrosine caused any additional chromatographic peaks. The parent compounds are not retained by Bio-Rez 70, but L-dopa causes a large increase in dopamine excretion, while α-methyltyrosine causes a small peak that elutes between norepinephrine and epinephrine and a large peak that is eluted after 14 min; we attributed these to α-methylnorepinephrine and α-methyldopamine, respectively. After treatment with α-methyltyrosine, patients' catecholamine values were in the low-normal range, as noted by others (2). Methenamine mandelate had not been taken by any of our subjects, but can be expected to interfere by reacting with catecholamines in urine. No interferences have been observed with samples from patients taking the following drugs in therapeutic doses: amiloride, amitryptiline, chlorothiazide, diazepam, digoxin, disopyramide, disodium chromoglycate, erythromycin, hydralazine, metoclopramide, metoprolol, nitrazepam, pindolol, pizotyline, prazosin, propranolol, pseudoephedrine, quinidine, salbutamol, and scopalamine.

One patient with pheochromocytoma was discovered during this study. For four consecutive urine samples, the norepinephrine excretion was between 290 and 450 μg/24 h. Plasma, sampled at various sites within the inferior vena cava, showed norepinephrine concentrations of 1.3 to 4.7 μg/L and suggested that the source lay approximately at the level of the renal veins. Radiographic examination disclosed an extra-adrenal tumor just below the right kidney, which on surgical removal was found to be a round mass of 40 to 50 mm diameter. Samples of it were assayed for catecholamines (9) and found to contain 1.0 to 1.6 mg of norepinephrine per gram of tissue, with negligible epinephrine and dopamine. One month after surgery this patient's norepinephrine excretion was 10 μg/24 h.

While not as sensitive as previous assays because of the large volume of resin eluate, the speed, simplicity, and freedom from significant interference make this procedure eminently suitable for routine use.

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References