Urinary Free Norepinephrine and Dopamine Determined by Reverse-Phase High-Pressure Liquid Chromatography

Leroy D. Mell and Anthony B. Gustafson

We used reverse-phase high-pressure liquid chromatography to measure free norepinephrine and dopamine simultaneously in human urine. Samples were treated with alumina, and the catecholamine(s) then eluted from it were directly injected onto a reverse-phase column (octadecyl-silica stationary phase), with 0.17 mol/liter acetic acid as the mobile phase and ultraviolet detection at 280 nm. The assay detects concentrations in urine as low as 5 pg/liter. Assay of 24-h urines (n = 10) gave within-run and day-to-day coefficients of variation of 3.7 and 4.7% for norepinephrine, and 2.6 and 3.5% for dopamine, respectively. Comparison studies with the traditional trihydroxyindole fluorometric method showed the liquid-chromatographic procedure to be more precise and subject to less interference. This relatively rapid procedure for urinary free norepinephrine and dopamine provides an efficient, reproducible method, readily adaptable to routine clinical use.

Additional Keyphrases: intermethod comparison - normal values - fluorometry - catecholamines

Increasing interest in catecholamine metabolism both in health and disease conditions (1–5) has led to an emphasis on developing reliable, sensitive methods for catecholamine determination. The trihydroxyindole fluorometric procedure (6–10) is now most commonly used to measure epinephrine and norepinephrine, but it is tedious, lacks precision, and is subject to dietary and drug interferences. Furthermore, dopamine must be determined by a still more difficult and time-consuming dihydroxyindole fluorometric procedure (11). Newer and more sensitive procedures include assays in which double-isotope derivative (12, 13) and radioenzymatic methods (14) are used.

An alternative approach to catecholamine analysis reported by Kissinger et al. (15) combines high-pressure liquid chromatography with electrochemical detection. This novel procedure is particularly suited for specimens of low catecholamine concentration or small volumes but necessitates the use of an electrochemical detector. More recently, Molnár and Horváth (16) have demonstrated the separation of catecholamines and related metabolites on a reverse-phase column (octadecyl-silica stationary phase) with aqueous, isocratic elution. They emphasized the importance of being able to separate acidic and basic compounds, and discussed in detail the effects of pH and ionic strength on catecholamine retention, without presenting evidence of clinical application. The purpose of this study is to demonstrate that free norepinephrine and dopamine in human urine can be determined quantitatively by reverse-phase chromatography with use of a simple aqueous, isocratic solvent system.

Materials and Methods

Apparatus

A Model 204 liquid chromatograph with models 440 UV absorbance detector, 6000A Solvent Delivery System, and U6K Universal Injector (Waters Associates Inc., Milford, Mass. 01757) was used for chromatography. An isocratic mode was employed, and the absorbance of the eluent was monitored at 280 nm with a Model 252A strip-chart recorder (Linear Instruments Corp., Costa Mesa, Calif. 92626). Chromatographic conditions are described in the figure legends. A Waters µBondapak C_{18} column (10-µm av particle size, 4 mm i.d. × 30 cm), used for all analyses, was flushed daily with “Spectrograde” methanol (Aldrich Chemical Co., Milwaukee, Wis. 53233). Fluorometric measurements were made with an Amino-Bowman spectrophotofluorometer with models P416-992 xenon lamp and 10-222 solid state photomultiplier microphotometer (American Instrument Co., Silver Spring, Md. 20910).

Reagents

In all experiments, 0.17 mol/liter acetic acid, pH 2.6, was used as the mobile phase. All solutions were made from de-ionized, glass-distilled water. The mobile phase was filtered through a 0.45-µm filter and degassed immediately before use.
Catecholamines and related metabolites were obtained from Calbiochem, La Jolla, Calif. 92037, and used as received. Standard solutions (1 g/liter) were prepared by dissolving these compounds in mobile phase. Working standards (10 mg/liter) were prepared by diluting standard solutions with mobile phase. Alumina (neutral, Brockman activity grade 1) was obtained from Sigma Chemical Co., St. Louis, Mo. 63178; before use it was acid-washed and stored in a desiccator.

Samples

Twenty-four-hour urine specimens were collected in dark glass containers from randomly selected and apparently healthy men; 10 ml of concentrated hydrochloric acid was used as the preservative. Four-hour urine specimens (0800 to 1200 h) were similarly obtained, with 2.5 ml of concentrated hydrochloric acid as preservative. Urine samples not immediately analyzed were stored at −35 °C for no longer than five days. Sample aliquots used for comparison of norepinephrine determinations by the trihydroxyindole and high-pressure liquid chromatography methods were obtained from the same alumina extraction of individual specimens, and assayed on the same day.

Procedure

Three grams of acid-washed alumina was placed in a 400-ml beaker containing 150 to 250 ml of urine and 5 ml of 40 mmol/liter disodium ethylenediaminetetraacetate. With slow stirring, we adjusted the pH to 7.5 with 5 mol/liter NaOH and then to pH 8.4 by further drop-wise addition of 1 mol/liter NaOH. The solution was stirred for 7 min at a speed just fast enough to keep the alumina suspended while 1 mol/liter NaOH was added as necessary to maintain pH 8.4. The alumina was then allowed to settle and the supernate decanted. The alumina was washed twice with 50 ml of water and quantitatively transferred to a chromatography column (1 cm i.d. × 18 cm; New England Nuclear, Worcester, Mass. 01608). The alumina was again washed twice with 10-ml portions of water. When the water level from the final wash reached the top of the alumina, 3 ml of 0.2 mol/liter acetic acid was added and the effluent void volume from this addition was discarded. Then two 5-ml portions of 0.2 mol/liter acetic acid were added to the column, yielding the catecholamine-containing effluent (10 ml). Aliquots from this collection were directly injected onto the reverse-phase column for high-pressure liquid-chromatographic determination. Fluorometric analyses of the aliquots with the Amino-Bowman spectrophotofluorometer followed procedures described by Crout (6) for norepinephrine and Bischoff and Torres (11) for dopamine. Results were arithmetically converted to 100% recovery based on recovery determined for standard solutions analyzed simultaneously.

Results and Discussion

Figure 1 shows the chromatogram obtained for a standard solution containing 20 ng each of norepinephrine, epinephrine, and dopamine in which the elution order reflects the polarity of these compounds (16), the most polar catecholamine being eluted first. For the chromatographic conditions shown in Figure 1, the limit of detection for each catecholamine is about 5 ng, or 0.1 mg/liter for a 50-μl sample volume—a value well below the expected concentration for norepinephrine and dopamine found in the alumina column eluent previously described. However, extracted urinary epinephrine concentrations are at or below this detection limit, making this analysis unfeasible with the present procedure (see Figure 3).

To quantitatively determine norepinephrine and dopamine, we compared individual peak heights to a standard curve (Figure 2). The abscissa in Figure 2 is expressed in absolute amount (ng) so sample volume injected could be varied. Such calibration curves for norepinephrine and dopamine are linear in excess of the range usually needed to determine urinary concentrations.

Although there are differences among urine specimens, the chromatogram depicted in Figure 3 is typical of those obtained after extraction with alumina. Compared to the chromatogram obtained by direct injection of an acidified urine sample onto a reverse-phase column (16), well-resolved peaks are obtained for norepinephrine and dopamine. Epinephrine, due to its low urinary concentration, is seen only as a small shoulder following the norepinephrine peak. At present two catecholamine metabolites, normetanephrine and metanephrine, have been identified. Additionally, L-dopa (not shown) can sometimes be observed as a small shoulder following the dopamine peak. For the specimens shown in Figure 3, the 24-h urinary excretions for norepinephrine and dopamine were 53 μg and 171 μg, respectively.

Trihydroxyindole Procedure Comparison

We determined urinary free norepinephrine excretion for 10 24-h specimens by both the trihydroxyindole and
An indication of the analytical superiority of the high-pressure liquid chromatography method is given by Table 2. While within-run chromatographic determinations for urinary norepinephrine and dopamine vary by 3.7% and 2.6% respectively, a higher coefficient of variation is observed for the trihydroxindole procedure. Results are similar on comparing the two methods for day-to-day determinations. Statistical variations of this magnitude for the trihydroxindole procedure can be anticipated because this method involves the differential estimation of norepinephrine and epinephrine by solving two simultaneous equations from data measured under different sets of conditions. In contrast, data obtained from the high-pressure liquid chromatography procedure can be converted directly to catecholamine concentration.

Drug Interference

Quantitative fluorometry of catecholamines is subject to interference from fluorescent drugs and catecholamine-like compounds, which may lead to spurious results (6). Although these interferences may be minimized by strict control of diet and abstinence from medications before sampling, such measures are not always possible. Many of the interferences can be eliminated by use of high-pressure liquid chromatography. Table 3 compares chromatographic results for

Table 1. Comparison of Normal 24-h Urinary Excretion of Norepinephrine and Dopamine, as Measured by High-Pressure Liquid Chromatographic and Fluorometric Methods

<table>
<thead>
<tr>
<th></th>
<th>Fluorometric</th>
<th>HPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Norepinephrine, µg/24 h</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crout (6)</td>
<td>30 ± 13(24)</td>
<td>—</td>
</tr>
<tr>
<td>Sourkes et al. (17)</td>
<td>40 ± 13(18)</td>
<td>—</td>
</tr>
<tr>
<td>Our laboratory</td>
<td>46 ± 21(78)</td>
<td>55 ± 14(10)</td>
</tr>
<tr>
<td><strong>Dopamine, µg/24 h</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sourkes et al. (17)</td>
<td>300 ± 63(18)</td>
<td>—</td>
</tr>
<tr>
<td>Barbeau et al. (18)</td>
<td>316 ± 72(24)</td>
<td>—</td>
</tr>
<tr>
<td>Our laboratory</td>
<td>235 ± 109(80)</td>
<td>271 ± 67(10)</td>
</tr>
</tbody>
</table>

*Mean ± SD(n).*

Table 2. Precision Data for Present (HPLC) Procedure for Norepinephrine and Dopamine and Trihydroxindole (THI) Procedure for Norepinephrine

<table>
<thead>
<tr>
<th></th>
<th>HPLC</th>
<th>µg/24 h THI</th>
<th>HPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Within-run, n = 5</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>82 ± 3</td>
<td>84 ± 15</td>
<td>307 ± 8</td>
</tr>
<tr>
<td>CV, %</td>
<td>3.7</td>
<td>18</td>
<td>2.6</td>
</tr>
<tr>
<td><strong>Day-to-day, n = 5</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>86 ± 4</td>
<td>80 ± 21</td>
<td>311 ± 11</td>
</tr>
<tr>
<td>CV, %</td>
<td>4.7</td>
<td>28</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Table 3 compares 24-h urinary free norepinephrine and dopamine data obtained in several laboratories by similar fluorometric methods. Table 1 additionally shows a comparison between these data and 24-h high-pressure liquid chromatography results for norepinephrine and dopamine.

High-pressure liquid chromatography procedures. The mean value ± SD for the trihydroxindole procedure was 58 ± 20 µg/24 h (range, 20 to 96 µg/24 h). The mean value ± SD by the high-pressure liquid chromatography method was 55 ± 14 µg/24 h (range, 27 to 82 µg/24 h). When values obtained by the high-pressure liquid chromatography method were regressed against those obtained with the trihydroxindole procedure, the slope of the linear least-squares line was 1.228, the intercept was -0.345, and the coefficient of correlation was 0.864. Table 1 compares 24-h urinary free norepinephrine and dopamine data obtained in several laboratories by similar fluorometric methods. Table 1 additionally shows a comparison between these data and 24-h high-pressure liquid chromatography results for norepinephrine and dopamine.
urinary free norepinephrine to data obtained by the fluorometric procedure in the presence of compounds known to interfere with it. These 4-h urines were from healthy individuals who were taking the indicated dosage. Spuriously low results were obtained by both methods when methyldopa mandelate was present. Formed by the metabolic breakdown of methyldopa mandelate in urine, formaldehyde inhibits the recovery of catecholamines during alumina extraction (19). As expected, three water-soluble vitamins that fluoresce did not interfere with the chromatographic procedure.

Of particular interest is the result obtained for methyldopa (Aldomet, Merck Sharp & Dohme). Methyldopa was not discontinued in the hypertensive patient studied, so the results are compared to a control value obtained from 11 healthy subjects. The trihydroxyindole procedure shows the expected spurious increase in urinary free norepinephrine excretion, caused by methyldopa interference (20). The high-pressure liquid chromatography method shows a decrease in excretion, apparently a result of the pharmacological action of methyldopa (21) rather than chemical interference. Methyldopa, L-dopa, dopamine, and norepinephrine may by readily identified in the same urine sample by high-pressure liquid chromatography because methyldopa, when present, is seen as a distinct peak following metanephrine. By such analyses, the chromatographic method may prove useful in further elucidating the action of this antihypertensive agent upon catecholamine metabolism.

In summary, the high-pressure liquid chromatography method for urinary free norepinephrine and dopamine is more precise and less subject to interference than is the traditional trihydroxyindole procedure. In addition, the relatively rapid analysis time of 5 to 10 samples per hour after alumina extraction makes the high-pressure liquid chromatographic method readily suited for routine clinical application.

This work was supported by the Naval Medical Research and Development Command, National Naval Medical Center, Department of the Navy, Research Task No. MF51.524.023.1007. The opinions and statements contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large. We thank Drs. David E. Uddin and F. Lee Rodkey, of the Naval Medical Research Institute, and Mr. Dave Skiles, of Waters Associates Inc., for their advice and assistance.

References