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Preparation of Urine Samples for Liquid-Chromatographic Determination of Catecholamines: Bonded-Phase Phenylboronic Acid, Cation-Exchange Resin, and Alumina Adsorbents Compared

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We compared results for the liquid-chromatographic determination of free norepinephrine and epinephrine in urine after purifying the catechols by the following methods: (a) acid-washed alumina, (b) weak cation-exchange resin (WCX), (c) a combination of weak cation-exchange resin followed by alumina (WCX-alumina), and (d) commercially available phenylboronic acid adsorbent. We evaluated analytical specificity, sensitivity, recovery, and turnaround time. The WCX-alumina combination produced the most sensitive and specific chromatograms for urinary catecholamines; the other methods took less processing time. Neither WCX nor alumina alone was suitable for routine work because of chromatographic interferences in a significant proportion of urines. The phenylboronic acid method is adequately sensitive and specific for norepinephrine and epinephrine, and samples can be assayed faster. Thus it provides a compromise between the high analytical performance of the WCX-alumina method and the speed of the WCX and alumina methods.

Additional Keyphrases: norepinephrine • epinephrine • cancer • pheochromocytoma • neuroblastoma • screening

"High-pressure" liquid chromatography is widely used for measuring total and free urinary catecholamines with either electrochemical or fluorometric detectors. In most electrochemical methods, the amines are isolated by a two-step procedure before injection into the chromatograph. Materials used for this include alumina, cation-exchange resins, and Sephadex (1–4). With each of these techniques the catecholamines are adequately separated from other urine components, but each is time consuming. Boric acid gels, which absorb compounds containing cis-diol groups, have been used recently to isolate urinary catecholamines (2, 5). Although these gels are highly specific towards catecholamines, they cannot be connected to a source of low pressure for rapid isolation and elution.

We evaluated a simultaneous dual-step purification procedure involving the use of chemically bonded materials (6) (ion-exchange and phenylboronic acid adsorbents: Bond Elut; Analytichem International, Harbor City, CA 90710) for rapidly isolating free catecholamines and compared results by this method with results obtained using weak cation-exchange resin and alumina.

Materials and Methods

Standards and reagents. Norepinephrine (NE), epinephrine (E), dopamine, and the internal standard, 3,4-dihydroxyphenylalanine, were from Sigma Chemical Co., St. Louis, MO 63178, as were all of the compounds in the interference study.1 Acid-washed alumina (Al2O3), prepared by the method of Anton and Sayre (7), was from Bioanalytical Systems, West Lafayette, IN 47905. The weak cation-

1 Nonstandard abbreviations: NE, norepinephrine; E, epinephrine; WCX, weak cation-exchange; PBA, phenylboronic acid; PSA, primary and secondary amine ion-exchange.
exchange (WCX) resin, Bio-Rex 70, was from Bio-Rad Laboratories, Richmond, CA 94804; it was prepared as described previously (3). The phenylboronic acid (PBA) and primary and secondary amine (PSA) bonded-phase columns were from Analytichem International.

Urine collection. We collected 24-h urine specimens in polyethylene containers, with concentrated HCl as preservative. The final pH of the urine was 2 to 3. Samples were stored at 2–8 °C before analysis.

Controls. We used normal and abnormal lyophilized urine controls (Ortho I and II; Ortho Diagnostic Systems, Raritan NJ 08869) for all determinations.

Apparatus. We used a Model 601 syringe-driven pump (Perkin-Elmer Corp., Norwalk CT 06856), a six-port rotary injector with a 20-μL sample loop (7125; Rheodyne, Cotati, CA 94928), a 4.6 mm × 10 cm strong cation-exchange analytical column (5 μm SCX; Analytichem International) with a reversed-phase C18 guard column (LiChrosorb RP-18; Brownlee Labs, Santa Clara, CA 95050), and an amperometric detector (Model LC-4B) containing a glassy carbon electrode (TL-5), both from Bioanalytical Systems, set at a potential of +650 mV vs Ag/AgCl. The mobile phase, phosphate buffer (100 mmol/L, pH 3.5) containing 100 mg of disodium EDTA per liter, was passed through a 0.22-μm pore-size filter (Millipore Corp., Bedford, MA 01730) before use. The vacuum manifold used (Vac-Elute) was from Analytichem.

Methods. For the combination of ion-exchange and alumina, we used the method described by Riggin and Kissing er (3). For ion-exchange (WCX) alone, we terminated the purification just before the addition of alumina, and injected the eluate. For purification with alumina alone, we adjusted 5.0-mL urine samples to pH 8.5 with 3 mol/L Tris buffer and added 50 mg of alumina, then proceeded as described (3) for the combination WCX-alumina method, a procedure similar to that described by Kissing er et al. (8).

For purification by PBA, we followed a method developed by Kabra and Dimson (unpublished work, 1984). In brief: condition the PBA column by washing with 1.0 mL of methanol and 1.0 mL of 0.1 mol/L HCl. Attach the PSA column above the PBA and condition in succession with 2.0 mL of methanol, 4.0 mL of NH4OH (3 mol/L) and finally 4.0 mL of 5 mmol/L phosphate buffer, pH 8.5. Adjust 1.0 mL of standard, control, or unknown urine to about pH 5.0 ± 0.5 with 0.3% NH4OH, add 75 μL of 1.0 mg/L dihydroxybenzylamine, and pour the mixture onto the PSA column. Allow the samples to flow through, then add 4.0 mL of pH 8.5 phosphate buffer. Rinse the column with an additional 2 mL of phosphate buffer, pH 8.5, and remove the PSA column. Now wash the PBA column (containing the catechols) with 1 mL of methanol, followed by 1 mL of an equilvolume mixture of acetonitrile and pH 8.5 phosphate buffer. Up until this point, all of the washes and the unadsorbed portions of the samples are discarded. Insert clean 200-μL polyethylene collection tubes into the vacuum manifold and elute the catecholamines with 1.0 mL of 0.1 mol/L HCl. Set the flow rate of the chromatograph to 1.0 mL/min. After equilibration, inject 20 μL of eluate into the chromatograph for analysis.

For all chromatographic methods the unknown catecholamine concentrations were determined by comparing peak heights for unknown urine with those produced by a calibrated urine pool containing known amounts of added NE and E. The peak heights were normalized with reference to the internal standard. The standard urine pool was calibrated by the method of standard addition. We kept aliquots of the standard urine pool at −20 °C until the day of use.

In addition to the above chromatographic methods, we estimated total urinary catecholamines, using fluorescence detection (9). We prepared urine samples by adsorbing the catecholamines onto alumina and eluting with acid (9). We then reacted the eluted amines with ferricyanide to form the trihydroxyindole derivative and monitored the resulting fluorescence without chromatographic separation at 490 nm after excitation at 405 nm.

Results

Precision, analytical recovery, and turnaround time. We determined within-run and day-to-day precision for the PBA and WCX-alumina purifications only, using five determinations for each catecholamine concentration in each study. Within-run CVs were 1% and 2% at a concentration of 45 ng of NE per milliliter and 6% and 4% at 310 ng of NE per milliliter for the PBA and WCX-alumina methods, respectively. For day-to-day precision, the CVs for NE were 6% and 7% at 45 ng/mL and 9% and 7% at 310 ng/mL, respectively. The CVs for epinephrine were slightly larger because its concentrations in these samples were lower. Both methods showed better reproducibility than the day-to-day precision obtained with the total fluorescence procedure: 15% and 14% for the low and high concentrations, respectively.

To assess analytical recovery, we added known amounts of NE and E to the calibrated pool and processed the urines by the various methods. The results for either norepinephrine and epinephrine were: PBA 80%, WCX 80%, alumina 80%, and WCX-alumina 60%.

The approximate time required for prechromatographic "cleanup" of 10 samples was: PBA 45 min, WCX 45 min, alumina 45 min, and WCX-alumina 120 min.

The lower recovery and longer turnaround time for the WCX-alumina method were the result of the additional steps involved.

Sensitivity. Figure 1 shows chromatograms for an aqueous solution containing 200 pg each of NE and E (with the usual amounts of the internal standard and dopamine) after treatment by each of the four cleanup methods. Because the dead volume of alumina is smaller than that of the bonded-phase columns and resins, one can elute the catechols from alumina with smaller volumes of eluent. Depending on the initial volume of urine sample and the final elution volume, the sample can be concentrated 10- to 25-fold. Thus, of the four methods tested, the alumina and WCX-alumina methods produce chromatograms with the greatest sensitivity. Samples can be concentrated fivelfold in the PBA method by eluting with lower volumes, but this decreases analytical recovery. Using the PBA method without concentration, we found a detection limit of about 50 pg for NE and 100 pg for E, sensitivities that suffice for the amounts ordinarily found in urine.

Specificity. Figure 2 shows typical chromatograms of urines analyzed by the four methods. Chromatographic interferences are observed for both WCX and alumina isolations, although the degree of interference varies from sample to sample. Based on these results, we conclude that neither WCX nor alumina is suitable for routine use without altering the chromatographic conditions. Both the PBA and the combined WCX-alumina methods produce chromatograms with minimal interferences in most cases.

Further to determine the specificity of PBA and WCX-alumina, we added several potentially interfering substances to normal urine (Table 1). Besides catecholamine metabolites and antihypertensive drugs, these included compounds with 3,4-dihydroxy functional groups, the portion of the catechol molecule responsible for adherence to the PBA column. None of these substances demonstrated...
interferences with the PBA or WCX-alumina methods under the liquid-chromatographic conditions described above. They either do not adhere to the adsorbent, do not oxidize at the selected potential, or have long retention times before they are eluted from the column.

**Correlation of results for patients.** Given the significant chromatographic interferences in the WCX and alumina methods, we did not attempt to correlate their analytical results. Figure 3 shows the correlation of total catecholamines \((NE + E)\) in 24-h urine with PBA vs WCX-alumina and PBA vs the non-chromatographic fluorometric assay. As shown, correlation between the two liquid-chromatographic methods is good, but poor between the PBA method and the fluorometric method for total catecholamines. In
Table 1. Compounds Not Interfering Chromatographically with the PBA and WCX-Alumina Methods

<table>
<thead>
<tr>
<th>Compound</th>
<th>Interference Computed Using the PBA Method</th>
<th>Interference Computed Using the WCX-Alumina Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metanephrine</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Normetanephrine</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Homovanilllic acid</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Vanillylmandelic acid</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>3-Methoxy-4-hydroxyphenylglycol</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>2-Methyl-3-(3,4-dihydroxyphenyl)alanylalanine</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>5-Hydroxyindoleacetic acid</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Deoxepinephrine</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Homogentisic acid</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>p-Dihydroxybenzolic acid</td>
<td>Positive</td>
<td>Positive</td>
</tr>
</tbody>
</table>

Fig. 3. Analytical correlation of results, after various prechromatographic treatments of patients' samples, for total catecholamines (NE + E) in 24-h urine.

Part, the differences reflect the poor specificity and precision of the total fluorometric method, samples with known interferences (e.g., methyl-DOPA) for the total fluorometric method having been included in this set.

Discussion

Reversed-phase columns such as C18 have become very popular in liquid chromatography because of their wide applicability, high efficiency, and excellent column stability and reliability. Consequently they have replaced ion-exchange columns in many catecholamine applications (10). Initially, we used a C18 analytical column and found it adequate for studies described above.

Recently, microparticulate (e.g., 5-μm particle size) chemically bonded ion-exchange materials have become available to overcome some of the earlier problems with reproducibility and stability. In evaluating the effectiveness of these new ion-exchange columns for urinary catecholamines, we found that ion-exchange columns had the advantage in the elimination of paired-ion reagents such as heptanesulfonic acid, which are often used to increase the retention time of catecholamines on reversed-phase columns. By obviating the use of these surfactants, we found less problems with bubbles in the flow cells, and equilibration times were shorter. Another advantage of ion-exchange columns is that back pressures are lower. We separated NE, E, dopamine, and the internal standard with adequate resolution within 10 min by using a combination of a C18 guard column coupled to a strong cation-exchange analytical column, and in general we encountered fewer chromatographic interferences. We conclude that an ion-exchange analytical column is better suited than a reversed-phase column for quantifying urinary catecholamines after PBA purification.

The use of a primary/secondary amine resin as a precolumn to the phenyboronic acid serves two purposes: it removes some potentially interfering amines that would be retained by the cation-exchange analytical column and detected if electrochemically active at +650 mV and it also increases the pH of the sample by adsorbing hydrogen ions. Samples need only be adjusted to pH 5.0 before passage through the PBA column, which will change the pH to 8.5. The samples so treated are immediately transferred to the PBA column for adsorption. Because the catecholamines are more stable at pH 5.0, there is less oxidative loss of the catechols than if the samples underwent more prolonged exposure to pH 8.5.

For the diagnosis of pheochromocytoma and neuroblastoma, either free or total urinary catecholamines can be used. Because these tumors are very rare, most results will be normal. Nevertheless, the mortality of undiagnosed cases mandates accurate and rapid screening methods. We have chosen to measure free urinary catecholamines because the time-consuming hydrolysis step is obviated. The sensitivity of the PBA method is such that no extra catechols need be added to the samples. In addition, the sensitivity in our detection system is improved by the use of a single-piston displacement-type pump, which produces pulse-free baseline. Nevertheless, the PBA method can be used satisfactorily with the more convenient and popular dual-piston reciprocating pumps. The major advantage of the PBA isolation system, especially when coupled to a vacuum manifold, is the shorter turnaround time so that more samples can be analyzed in a work day.

References

Laboratory Evaluation of the Greiner G-400 Discrete Selective Multichannel Analyzer

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We evaluated the new Greiner G-400 selective multichannel analyzer. Thirty different tests can be run in combination profile and single-test mode at a rate between 150 and 300 tests per hour. The new photometer allows kinetic and equilibrium (endpoint) measurements. The G-400 analyzer demonstrated excellent precision, linearity, accuracy, ease of operation, and no carryover. Results for 16 different analytes as determined with the Hitachi 705, GSA II, and Corning 940 correlated favorably with those obtained with the G-400. The G-400 analyzer is suitable for both emergency and routine use.

The G-400 analyzer is a discrete, selective multichannel analysis system designed for determining enzyme activities and concentrations of substrates and electrolytes. We evaluated the performance of the analyzer for 16 different analytes, and compared the results with those obtained with other equipment in our routine laboratory.

Materials and Methods

Instrumentation

We compared the Greiner G-400 analyzer (Greiner Instruments AG, 4900 Langenthal, Switzerland) with the former Greiner Selective Analyzer, the GSA II (J). The newly developed analyzer differs in many respects:

- The capacity varies from 150 to 300 tests per hour, depending on the use of a sample blank or a reagent blank, respectively. Only one sample dispenser is used for sample volumes between 5 and 100 μL. The process tubes are reusable after washing, but must be replaced after 10 000 tests. Handling of the 30 reagent dispensers is simple. Their dead volume can be minimized to 5 mL. The G-400 is equipped with a double-beam filter photometer (eight wavelengths). The light of the mercury lamp is split into two beams. A thermostated carrousel with eight quartz cuvettes passes through both light beams. The rotating filter wheel allows it to monitor 10 readings at 1-s intervals for each beam and wavelength. With this procedure, kinetic measurements can be performed (Figure 1).

- Software controls the linearity of reactions and aberrant absorbances (e.g., hemolysis, hyperlipemia) and compares results with reference values. A microprocessor can handle the absorbances of as many as four wavelengths per test, allowing polychromatic measurements (optional). The G-400 can be integrated in a laboratory information system by a bidirectional interface, RS 232. If the data transfer to the periphery is interrupted, up to 300 results can be stored in the output data buffer to prevent their loss.

- We compared results with those obtained with the Hitachi 705 (Boehringer Mannheim AG, 6343 Rotkreuz, Switzerland), the GSA II (Greiner Instruments AG), and the Corning 940 Calcium Analyzer (Coramed AG, 8305 Dietikon, Switzerland) instruments.

Control Materials and Specimens

We used the following control sera: "Moni-trol II" (lot 65), "Enza-trol" (lot 251), and "Lab-trol" (lot 105-9), all from Merz & Dade AG, 5186 Düdingen, Switzerland, and "Seronat..."