Coupled-Column Liquid Chromatographic Analysis of Catecholamines, Serotonin, and Metabolites in Human Urine

TORSTEN J. PANHOLZER, JÜRGEN BEYER, and KLAUS LICHTWALD*

A column-switching HPLC system was utilized for the simultaneous determination of epinephrine, norepinephrine, dopamine, serotonin, and their metabolites metanephrine, normetanephrine, 3,4-dihydroxyphenylacetic acid, homovanillic acid, and 5-hydroxyindoleacetic acid in human urine. The sample was injected directly onto a C18-alkyl-diol silica precolumn, which separated the analytes from matrix. The analytes were eluted from the precolumn onto the analytical column by the use of column-switching techniques and were then separated on the analytical column by means of ion-pair reversed-phase HPLC. The analytes were then oxidized to the corresponding quinones and converted into fluorescent derivatives by reaction with meso-1,2-diphenylethylenediamine.

The concentrations of catecholamines and their metabolites in urine reflect the activity of the sympathoadrenal system. The quantification of these compounds is important for the diagnosis of pheochromocytoma and related diseases (1-4). Of the patients in whom pheochromocytoma is found at autopsy, <50% had been diagnosed with the disease while they were alive (5). The lack of a definitive laboratory test for the disease might be one reason. There is still no general agreement as to which analytes other than epinephrine (E)1 and norepinephrine (NE) have the most diagnostic importance. Several investigators favor the acidic metabolites (6, 7), whereas others recommend the metanephrines (8, 9). Most published analytical methods determine only some of the important compounds in a simultaneous measurement (9, 10). It would be advantageous if more of them could be analyzed within a single assay, which would include sample pretreatment and chromatographic analysis.

We here present a method that permits injection of an unprocessed urine sample and the simultaneous measurement of E, NE, dopamine (DA), metanephrine (M), normetanephrine (NM), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA), as well as serotonin (5HT) and 5-hydroxyindole-3-acetic acid (5HIAA). System-integrated sample processing was achieved by the use of a restricted access silica precolumn device. The precolumn is coupled by an electrically driven valve to an analytical column on which the analytes were chromatographed. Ion-pair reversed-phase chromatography was used as separation mode, and was followed by postcolumn derivatization and fluorometric detection1.

**Materials and Methods**

**APPARATUS**
A schematic diagram of the liquid chromatographic system is shown in Fig. 1. We used the model 2700 Solvent Delivery System (Bio-Rad), which consisted of two dual piston HPLC pumps (pumps 1 and 2 in Fig. 1). The Hitachi reaction pump (model 655A-13; Merck) has three parallel pump heads for feeding three reagents, two were used as pumps 3 and 4. Injections were made with a Rheodyne valve (model 7125) equipped with a 100-µL sample loop. The LiChrospher RP-18 ADS precolumn (particle size, 25 µm; column size, 25 × 4 mm i.d.; Merck) was connected via an electrically driven six-port switching valve (WE C6WK; Valco Europe, distributed by Merck) to the analytical reversed-phase column (LiChrospher 100 RP-18; particle size, 5 µm; column size, 125 × 4 mm i.d.; Merck). The coils in the derivatization unit were made of Teflon (0.33 mm i.d.). Coils a and b were 2 and 7 m long and were heated in a column oven (LaChrom L7350; Merck) at 95 °C. Coil c was 1.2 m long and was

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1 Nonstandard abbreviations: E, epinephrine; NE, norepinephrine; DA, dopamine; M, metanephrine; NM, normetanephrine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; 5HT, serotonin; 5HIAA, 5-hydroxyindole-3-acetic acid; and DPE, meso-1,2-diphenylethylenediamine.
placed together with the analytical column in a thermostated water bath (type D1+G; temperature range, −10 to 100 °C; Haake) at 10 °C. The fluorescence emissions of the analytes were measured at 480 nm emission, with excitation at 350 nm, by a Hitachi fluorescence spectrophotometer (model F-1050; Merck) equipped with a 12-μL flow cell (cat no. 050-1322). The High Resolution Liquid Chromatographic System interface (model 822; Bio-Rad) controlled the switching valve and pumps 1 and 2. In addition, it transferred the data from the detector to the Bio-Rad software (Series 800 High Resolution Liquid Chromatographic System, Ver. 2.30.1a), which monitored the chromatograms and determined the areas under the detected peaks.

CHEMICALS

DA was obtained from Serva; DOPAC and HVA were purchased from ICN; and 5HIAA, M, NE, NM, and 5HT were from Sigma-Aldrich. E and the other chemicals were at least of analytical reagent grade and were supplied by Merck. meso-1,2-diphenylethlenediamine (DPE) was synthesized by the procedure of Irving and Parkins (11).

SOLUTIONS

The mobile phase for both columns was a buffer solution containing 0.1 mol/L sodium dihydrogen phosphate monohydrate, 5 mmol/L sodium octyl sulfate, and 0.1 mmol/L sodium azide. The pH was adjusted to 2.5 with orthophosphoric acid (200 mL/L). The oxidizing reagent was an aqueous solution composed of 20 mmol/L sodium periodate and 6 mmol/L potassium hexacyanoferrate (III). The fluorescence reagent was 700 mL/L ethanol solution containing 60 mmol/L DPE and 0.3 mol/L sodium hydroxide. The deionized water used for all methods was purified by the Millipore reagent grade water system (MilliQ ZFMQ 23004). All solutions were degassed for 5 min in an ultrasonic bath before use. The stock solution used for calibration contained 10⁻⁴ g/L DA, E, and NE, and 10⁻³ g/L M, NM, DOPAC, HVA, 5HT, and 5HIAA. All calibrators were constituted from 1 g/L stock solutions in mobile phase buffer.

URINE SPECIMENS

Urine specimens (24 h) were collected and acidified with 2.5 mol/L sulfuric acid containing 100 g/L glycine (10 mL/L urine). Aliquots (10 mL) were stored at −20 °C. Before injection, the urine was centrifuged at 1200g for 10 min at room temperature.

To assess imprecision specimens from 20 normotensive and hypertensive adult individuals were measured. The samples used had previously shown no pathological catecholamine concentrations according to the method of Kringe et al. (12).

Aliquots (10 mL) from 113 individual nonpathological 24-h urine samples were pooled for use as the in-house control.

Three single specimens from patients with pheochromocytoma confirmed by surgery were measured.

For method validation, two control urines were obtained from Chromsystems, one with physiological (control urine I) and the other with pathological concentrations (control urine II) of the relevant analytes and contained the following concentrations: control urine I, 66 μg/L NE, 13 μg/L E, 265 μg/L NM, 129 μg/L M, 180 μg/L DA, 3.4 mg/L DOPAC, 4.2 mg/L HVA, 141 μg/L 5HT, and 5.5 mg/L 5HIAA; control urine II, 213 μg/L NE, 50 μg/L E, 1.1 mg/L NM, 301 μg/L M, 525 μg/L DA, 11 mg/L DOPAC, 15 mg/L HVA, 0.9 mg/L 5HT, and 31 mg/L 5HIAA.

PROCEDURE

Sample was applied when the valve was in position A (Fig. 1). The mobile phase, delivered by pump 1 at a flow rate of 0.3 mL/min, eluted mainly the high-molecular weight and hydrophobic components of the sample from the ADS precolumn to waste. Simultaneously, the analytes were retained on the hydrophobic bonded phase of the sorbent. Five minutes after sample injection, the six-port valve rotated 60° to position B, coupling the precolumn to the analytical column, and the mobile phase, delivered by pump 2 at a flow rate of 0.8 mL/min, eluted the analytes from the precolumn. The precolumn and the analytical column were coupled for 11 min to allow transfer of the analyte fraction onto the analytical column, after which the valve switched back to position A and separation on the analytical column continued. Methanol (200 mL/L) was added to the mobile phase 110 min after injection to accelerate the elution of the last analyte (5HT). During the whole procedure, pumps 3 and 4 of the derivatization unit introduced the oxidizing and fluorescence reagents to the column eluate at a flow rate of 0.15 mL/min. The operating sequence is listed in Table 1.
CALCULATIONS
To calculate analyte concentrations, the areas under the peaks of unknown samples were related to the peaks of the stock mixture used as calibrator. The linear regression data were processed by the program Microcal Origin, Ver. 4.10 (Additive).

RESULTS
Representative chromatograms of the stock mixture, a nonpathological 24-h urine, and urine from a patient with pheochromocytoma confirmed by surgery are shown in Fig. 2. Nine compounds could be separated within 150 min, including sample pretreatment. The shift of the baseline at 110 min is the result of the isocratic introduction of the mobile phase containing 200 mL/L methanol.

SAMPLE PRETREATMENT
The ADS column, which permit a system-integrated sample processing, was developed by Boos et al. (13); to our knowledge, the present study was the first time it was used to separate catecholamines and their metabolites within a single analysis. This porous alkyl-diol silica (LiChrospher RP-18 ADS) consists of a hydrophilic and electroneutral external particle surface and a hydrophobic reversed-phase internal surface. These bimodal chromatographic properties allow retention of hydrophobic low-molecular weight analytes by classical reversed-phase chromatography exclusively at the hydrophobic pore surface. Macromolecular constituents of the sample matrix are size-excluded by 6 nm pores and eluted into the waste.

Online analysis was performed by coupling of the ADS-precolumn and the analytical column via an electrically driven six-port valve. The switching of the valve to position B ended sample pretreatment on the precolumn and coupled it with the analytical column. The switching point was programmed at 5 min after injection. At this time point, most of the sample matrix, monitored by a detector (wavelength 280 nm) in a previous test analysis, had eluted from the ADS; however, NE, the first analyte to elute from the analytical column, was still retained. The change in flow direction (back flush) after the valve was switched achieved a transfer of the concentrated analytes as a single band to the analytical column. This was monitored by an in-line detector (wavelength, 280 nm) between the precolumn and the analytical column. Eleven minutes after the valve switched to position B, it switched back to position A.

SEPARATION OF THE ANALYTES
The catecholamine-related compounds contain amino, carboxyl, and alcohol groups in their molecular structure. For the simultaneous separation of such analytes, ion-pair reversed-phase HPLC is the method of choice. Separation was achieved with the mobile phase described with 5 mmol/L sodium octyl sulfate added as the ion-pair reagent. Concentrations of sodium octyl sulfate as high as 5 mmol/L were essential for maintaining constant retention times, especially for late-eluting analytes.

Table 1. Operating sequence.

<table>
<thead>
<tr>
<th>Time, min</th>
<th>Valve position</th>
<th>Methanol content of mobile phase, mL/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inject</td>
<td>A</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>B</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>A</td>
<td>0</td>
</tr>
<tr>
<td>110</td>
<td>A</td>
<td>200</td>
</tr>
<tr>
<td>End</td>
<td>A</td>
<td>200</td>
</tr>
</tbody>
</table>

Fig. 2. Chromatogram of the stock mixture (A), a human nonpathological 24-h urine (B), and urine from a patient with pheochromocytoma confirmed by surgery (C).

(A), the shift of the baseline at 110 min is a result of the addition of 200 mL/L methanol to the mobile phase. (C), values shown in Table 5, patient 3.
In addition to the LiChrospher 100 RP-18 column, we tested three other C18 reversed-phase columns: one column packed with Nucleosil 100–5 C18 (200 × 4 mm; Machery & Nagel), one column “for catecholamine analysis”, but not otherwise specified (100 × 4 mm; Chromsystems), and one column packed with SilicaROD RP-18 (100 × 3.4 mm; Merck, Darmstadt, Germany). The last column showed a pressure at the pumps that was reduced to 27%, but the LiChrospher column gave the best resolution.

A constant column temperature is important for the analysis. A linear relationship between the retention times of the catecholamine-related compounds and the column temperature was found. At low temperatures, resolution of the peaks was higher, retention times were increased, and peak broadening was reduced. The decision was made to use the analytical column at 10 °C.

**POSTCOLUMN DERIVATIZATION**

Postcolumn derivatization was based on the method by Jeon et al. (14). In this reaction, the catecholamine-related compounds are first oxidized with periodate and potassium hexacyanoferrate to the corresponding o-quinones. Those activated molecules form with the introduced DPE via azomethines to 2-phenylbenzoxazole derivatives, which show fluorescence at 480 nm after excitation at 350 nm (15). We found that 5HT and 5HIAA also show fluorescence under these conditions, although they could not be oxidized to o-quinones, which are necessary intermediates for the formation of 2-phenylbenzoxazole derivatives. Their signals were reduced to 13% compared with that of E on a molar basis. Without the oxidizing reagent and DPE, none of the analytes, in physiological concentrations, fluoresce at the 480 nm with the chosen detection gain.

For the derivatization unit, we used different materials than did Jeon et al. (14). We tested the length of the reaction coils and found that lengths of 2 m for the oxidation and 7 m for the fluorescence reaction offered the best compromise between peak height and peak width. The coils were heated in a column oven at 95 °C.

The apparent temperature of the eluate stream was 48 °C. E, with the lowest concentration of the nine analytes in urine under physiological conditions, produced maximum peaks at this temperature. A DPE concentration of 0.06 mol/L and an apparent pH 6.0 in the fluorescence reaction coil, which is achieved with 0.3 mol/L sodium hydroxide in the fluorescence reagent, were the most favorable for the condensation and cyclization reactions. Before reaching the detector, the eluate passed through the 1.2-m coil in a cooling bath (10 °C) and was cooled to 19 °C.

**VALIDATION**

**Identification.** We identified the peaks in urine by monitoring the coincidence of the retention times and the increases in peak heights when stock solutions of the calibrators were cochromatographed with the urine samples. The analytes showed no peaks when DPE and the oxidizing reagents were omitted from the solutions for the derivatization.

**Linearity and sensitivity.** Injections of stock solutions in the concentration range $1 \times 10^{-6}$ to $1 \times 10^{-2}$ g/L indicated that the monitored signals were linear for each analyte.
The linear regression data are presented in Table 2. The slopes of the regression lines are the measures for the analytical sensitivity.

**Precision.** Intra- and interassay imprecision as determined from analysis of the stock mixture and control urines I and II, is summarized as CVs in Table 3. The concentrations of the analytes in each of the control urines are listed in Materials and Methods.

**Recovery and agreement with expected values for control urines.** The analytical recoveries are shown in Table 4. For determination of the matrix-independent recovery, the stock mixture was chromatographed 10 times with ADS sample pretreatment and 10 times without ADS. The values without pretreatment were set as 100% and the values with pretreatment were related to them. Therefore, the matrix-independent data describe the influence of sample pretreatment on the recovery for the stock mixture. For the matrix-dependent recovery, known amounts of stock solutions at three different concentrations were added (each three times) to control urine I and analyzed. The concentrations of analytes in the stock solutions and urine were also measured separately in single analyses. The results of the cochromatographed samples were related to the sum of the single samples. Therefore, the matrix-dependent data describe the influence of the sample matrix on recovery. In addition, we compared the values of control urine I specified by the manufacturer with the concentrations determined (n = 20). The deviations from the expected values are also summarized in Table 4.

**Comparison of methods.** The measured values for E and NE in 27 urine samples from different individuals were compared with those determined with the method of Kringe et al. (12). Their HPLC procedure included extraction by aluminum oxide, reversed-phase separation, and fluorescence detection of the trihydroxyindole derivatives, and determined only E and NE. The correlation of E and NE measured with both methods is 97.5% and 96.6%, respectively (Fig. 3).

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**Table 4. Recoveries, deviations from expected values, and detection limits.**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Recovery, %</th>
<th>Deviation, %</th>
<th>Detection limit, g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>NE</td>
<td>88</td>
<td>88</td>
<td>$-2.6$</td>
</tr>
<tr>
<td>E</td>
<td>94</td>
<td>88</td>
<td>$-12$</td>
</tr>
<tr>
<td>NM</td>
<td>95</td>
<td>95</td>
<td>$-6.1$</td>
</tr>
<tr>
<td>M</td>
<td>96</td>
<td>86</td>
<td>$-12$</td>
</tr>
<tr>
<td>DA</td>
<td>97</td>
<td>90</td>
<td>$-2.6$</td>
</tr>
<tr>
<td>DOPAC</td>
<td>95</td>
<td>92</td>
<td>$-7.6$</td>
</tr>
<tr>
<td>HVA</td>
<td>89</td>
<td>84</td>
<td>$-0.1$</td>
</tr>
<tr>
<td>5HT</td>
<td>87</td>
<td>82</td>
<td>$+4.4$</td>
</tr>
<tr>
<td>5HIAA</td>
<td>96</td>
<td>91</td>
<td>$+0.4$</td>
</tr>
</tbody>
</table>

$^a$ Signal-to-noise ratio = 3.

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Fig. 3. Comparison of methods.

Urine samples (n = 27) of individuals were measured with the described method and the method of Kringe et al. (12). The values of E (▼) and NE (■) determined for each sample are shown. Dotted lines represent the maximum correlation; solid lines represent the actual regression. The regression equation for E is: $y = 0.93x + 1.0 (r = 0.975)$; and for NE is: $y = 0.88x + 35.3 (r = 0.966)$. 

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Detection limits. The detection limits for the analytes at a signal-to-noise ratio of three were based on the linear dilution of their stock solutions (Table 4).

Values in subjects with and without pheochromocytoma. Each analyte was determined in single 24-h urine samples collected from 20 normotensive and hypertensive adult individuals (Table 5). In addition, we measured a pooled urine that contained aliquots of 113 24-h urines. Previously, the E and NE in each of the 20 and the 113 samples were measured by the method of Kringle et al. (12) and showed E and NE values that were not increased. With the new method, the pooled urine was assayed 15 times. The means of the obtained concentrations were related to the average volume of the 113 urines. Those results are near the mean values for the 20 individuals and are shown in Table 5 contrasted to the results from 3 patients with confirmed pheochromocytoma. Patient 2 is one case of a group whose in 24-h urines showed increased E but NE within the reference interval. Patient 3 is a single case with additional high 5HT values.

Discussion

Because of the diversity of catecholamine-secreting tumors, a complete analysis of the catecholamines and their metabolites is clinically desirable in several cases. Different methods for determining these analytes in urine with a single HPLC-method have been attempted (9, 10, 16, 17); however, most of the procedures require tedious manual sample pretreatment using ion-exchange resins. In these procedures, the analytes are often diluted, and interfering components are not sufficiently reduced. We developed a specific, sensitive, and automated coupled-column HPLC method that determines simultaneously the three catecholamines, the metanephrines, and two metabolites of DA, as well as serotonin and its metabolite 5HIAA. This is the first time to our knowledge that nine analytes and transmitters and their metabolites in urine were estimated within a single analysis under uniform chromatographic conditions, which widens the diagnostic possibilities for pheochromocytoma and related diseases, especially for more complex entities.

Simple system-integrated sample processing was achieved with a restricted access precolumn (ADS). When column-switching techniques were used, the analytes were eluted from the ADS precolumn to the analytical column without dilution and external transfer. This is documented in the nearly quantitative matrix-independent recovery (Table 4). The size exclusion of the precolumn eliminated many of the compounds that interfere with the separation or contaminate the analytical column. This effect in combination with the postcolumn derivatization leads to a selective analysis.

The compounds were separated by means of ion-pair reversed-phase chromatography. The conditions for the separation were similar to those mentioned in the literature (9, 16, 17). A C18 column was used as stationary phase, and a buffer at pH 2.5 with the ion-pair reagent sodium octyl sulfate and an organic modifier was used as the mobile phase. Contrary to the specifications of several investigators (10, 14, 16, 17), we could not achieve constant retention times with concentrations of the ion-pair reagent lower than 5 mmol/L.

The postcolumn derivatization was based on the method of Jeon et al. (14). However, we used reaction coils with smaller internal diameters, which maintained the high pressure during derivatization and prevented the generation of bubbles, especially in the heated areas. In addition, the use of coils with smaller internal diameters reduced peak broadening. Our oxidizing and fluorescent reagents were, in contrast to those of Jeon et al., twice as concentrated but were introduced at flow rates 50% lower than the flow rates recorded in that study. This caused less dilution of the separated analytes in the coils. Before the eluate reached the fluorescence detector, its temperature was reduced by a cooling coil in a thermostated bath. Jeon et al. used only an air-cooled coil. Effective cooling is important for holding the flow cell near room temperature. Furthermore, a high solvent temperature causes a decline in fluorescence intensity.
The DPE-dependent fluorescence of 5HT and its metabolite 5HIAA was observed at 480 nm. Although they do not undergo conversion to benzoxazole derivatives, they are suitable informative analytes in the diagnostic field of disturbed neurotransmitter secretion (Table 5).

More peaks are separated in the chromatograms of urine than the defined analytes. These unidentified compounds, which show DPE-dependent fluorescence, have yet to be described.

The detection limits of the method are sufficient for urinary measurements. To date, the low concentrations of these analytes seen in plasma samples cannot not be detected by this method. The values for urine specimens agree with reference intervals determined with other HPLC methods (1, 2, 18). The values for the pooled urine sample from 113 selected normotensive and hypertensive adults were within the ranges for the 20 subjects without pheochromocytoma (Table 5).

We thank K.-S. Boos for informative discussions and helpful advice in a critical phase of method development. We also thank D. Lubda (Merck, Darmstadt, Germany) for the supply of a new generation of columns.

References