Determination of Metanephrines in Plasma by Liquid Chromatography with Electrochemical Detection

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Metanephrines are O-methylated metabolites of catecholamines. We report the use of liquid chromatography with electrochemical detection to determine plasma concentrations of normetanephrine (NMN) and metanephrine (MN). Plasma NMN and MN in 32 normal volunteers and inpatients were compared with concentrations in 23 patients with pheochromocytoma. Metanephrines were adsorbed from plasma onto a cation-exchange column and eluted with ammoniacal methanol. The dried residue was dissolved in mobile phase and injected onto a reversed-phase column. Recoveries of NMN and MN from 1 mL of plasma averaged 50–70%, and results varied linearly with quantity injected over a range of 0.13–55 pmol. The detection limit was 25 fmol for NMN and 50 fmol for MN. Intra-assay CVs were <5%. In normal volunteers and inpatients, plasma concentrations of NMN ranged between 0.12 and 0.73 nmol/L (mean 0.38 nmol/L), and MN between 0.06 and 0.63 nmol/L (mean 0.19 nmol/L). Plasma NMN concentrations were increased in all 23 patients with pheochromocytoma (range 1–172 nmol/L), whereas MN concentrations (range 0.10–382 nmol/L) were increased in only 9 patients. The assay method is reliable and sensitive and offers an approach to examine the extraneuronal metabolism of catecholamines. The method may also be useful in the diagnosis of pheochromocytoma.

Additional Keyphrases: chromatography, reversed-phase, catecholamine, pheochromocytoma

Normetanephrine (NMN) and metanephrine (MN), O-methylated metabolites of norepinephrine and epinephrine, are produced by the actions of catechol-O-methyltransferase (EC 2.1.1.6), an enzyme largely confined to extraneuronal tissues (I). Measurements of NMN and MN concentrations in biological fluids may therefore provide a means to examine extraneuronal metabolism of catecholamines.

Liquid chromatography and gas chromatography/mass spectrometry methods have been described for determining metanephrine concentrations in urine (2–5) and brain tissue (6, 7). Because of the low concentrations of NMN and MN in plasma, measurement of metanephrines in this fluid matrix has been more difficult than in urine and tissue. Adequate sensitivity for measurement of plasma NMN has been achieved with a radioenzymatic technique (8), but this method does not measure plasma MN. Three liquid-chromatographic procedures for measurement of plasma metanephrines have been described (9–11). Two yielded unrealistically high plasma concentrations of NMN (9, 10), but estimates with the third, more recent, procedure (11) agreed with those determined by the radioenzymatic method (8). Although the sensitivity of this last chromatographic method was sufficient for measuring total plasma metanephrines (free and conjugated), the amount of free metanephrines in 1 mL of plasma was close to the detection limit of that assay.

Here we describe a method utilizing liquid chromatography with electrochemical detection, with adequate sensitivity for reliable measurement of both NMN and MN in 1 mL of plasma. Plasma concentrations measured in normal volunteers and in hospital inpatients were compared with those in patients with pheochromocytoma, a rare tumor that secretes catecholamines into the blood stream.

Materials and Methods

Materials

Apparatus. To extract NMN and MN from plasma, we used an AccuCAT™ silica solid-phase cation-exchange column (200- or 600-mg bed volume) in combination with a Vac-Elut® SPS 24 vacuum manifold (both from Analytichem International, Harbor City, CA). Eluates were lyophilized by using a SVC200H Speed Vac Concentrator and an RT490 refrigerated condensation trap (both from Savant Instruments, Farmingdale, NY).

The apparatus for liquid chromatography included a Model 590 solvent-delivery system and a Model 710B WISP autosampler (both from Waters Associates, Milford, MA) on line to a 5-μm particle size, 4.6 × 250 mm, C18 analytical reversed-phase column (P/N 235329; Beckman Instruments, San Ramon, CA). The column temperature was maintained at 13 °C by using a refrigerated water circulator (Lauda K-2/R; Brinkmann Instruments, Westbury, NY) that pumped a chilled equilibrium mixture of water and ethylene glycol through a water jacket (P/N 250-13; Beckman Instruments) surrounding the column.

A Model 5100A coulometric detector (Environmental Sciences Associates, Bedford, MA) with a triple elec-
trode system was used to quantify the compounds. The first electrode in the Model 5021 conditioning cell was set at an oxidizing potential of +0.39 V; the second and third electrodes in the Model 5011 analytical cell were set at potentials of +0.15 V and −0.39 V. The output from the third electrode was recorded with a Macintosh Classic II computer (Apple Computer, Cupertino, CA) and Dynamax Method Manager software package (version 1.3; Rainin Instruments, Woburn, MA).

**Standards.** Six compounds were chromatographed routinely as standards: the O-methylated metabolites of the three endogenous catecholamines norepinephrine, epinephrine, and dopamine and three structurally similar compounds evaluated as internal standards (Figure 1). The hydrochloride salts of MN and NMN were obtained from Sigma Chemical Co. (St. Louis, MO). The hydrochloride salt of the O-methylated metabolite of dopamine, 3-methoxy-4-hydroxyphenethanolamine (MHPEA), was obtained from Research Biomedicals Inc. (Natick, MA). A candidate for the internal standard of the assay, 4-hydroxy-3-methoxybenzylamine (HMBA), was obtained as the hydrochloride salt from Aldrich Chemical Co. (Milwaukee, WI). Two other candidates for internal standards, 3-ethoxy-4-hydroxyphenylethanolamine (EHPEA) oxalate and 6-fluoronormetanephrine (FNMN), were provided by Kenneth L. Kirk, who synthesized the compounds according to previously published procedures (4, 12). Standards were stored in 0.2 mol/L acetic acid in 1-mL aliquots at −70 °C.

**Mobile phase.** The mobile phase (per liter, 0.1 mol of NaH₂PO₄, 0.13 mmol of EDTA, 0.34 mmol of sodium octane sulfonate, and 65 mL of acetonitrile) was adjusted to pH 3.4 with phosphoric acid (850 g/L), filtered through a 0.22-μm pore-size filter, and degassed before it was pumped through the chromatographic system at a rate of 1.0 mL/min.

**Blood Samples.** Antecubital venous blood was collected from 14 healthy normal volunteers, 18 hospital inpatients who were admitted for diagnostic cardiac catheterization, and 23 patients with a histologically proven pheochromocytoma. In the patients with pheochromocytoma, venous plasma norepinephrine ranged from 2.8 to 1360 nmol/L (mean 103 nmol/L) and plasma epinephrine from 0.03 to 1111 nmol/L (mean 70 nmol/L). Samples from the normal volunteers were collected while the subjects were semirecumbent, whereas those from the patients were taken with the subjects supine. Heparinized samples were placed on ice and the plasma was separated by refrigerated centrifugation and stored at −70 °C. Thawed plasma samples were centrifuged before extraction.

**Procedures.**

**Extraction.** To activate the solid-phase cation-exchange matrix, we passed through the extraction columns 15 mL of a 3/1 (by vol) mixture of a 10-fold dilution of concentrated ammonia and methanol (ammoniacal methanol), followed by 2 mL of 10 g/L potassium hydroxide in methanol. The column was then washed with 2 mL of de-ionized water. During these procedures, the suction applied was regulated carefully to prevent drying the column-bed. We then diluted 1 mL of plasma (or 1 mL of de-ionized water as a blank) with 4 mL of de-ionized water and added 1 ng of each of the internal standards (HMBA, EHPEA, and FNMM). The diluted sample was passed through the column at a flow rate of 2 mL/min. We rinsed the column sequentially with 5 mL of 10 mmol/L acetic acid plus methanol (9/1 by vol), 5 mL of 10 mmol/L ammonium phosphate (pH 8), and 5 mL of de-ionized water. Metanephrines were eluted from the column with 2 mL of ammoniacal methanol and collected in a glass tube. The eluate was evaporated by centrifugation under reduced pressure, and the residue was dissolved in 150 μL of mobile phase, of which 140 μL (or less for pheochromocytoma patients) was injected onto the column.

**Assay validation.** We examined the detector response relative to amounts of injected NMN and MN standards and as functions of oxidizing potential or mobile phase pH. Limits of detection were calculated as the amount of injected NMN or MN that gave a detector response threefold greater than the baseline fluctuation. Recoveries of NMN and MN from water and from plasma (to which known amounts of standards were added) were compared with recoveries of each of the three internal standards. Concentrations of NMN and MN in plasma were calculated by correction for loss during extraction by use of the analytical recovery of the internal standard that had the closest recovery to NMN and MN.
Intra-assay CVs were determined by measuring plasma NMN and MN in multiple pooled plasma samples that were extracted and assayed over the course of 1 day. Interassay CVs were assessed by repeated measurements of plasma NMN and MN in pooled plasma samples on separate days. Intra- and interassay CVs were then determined for aliquots of a pooled plasma sample drawn from healthy subjects at rest (low normal range) and in a pooled sample from subjects after moderate exercise (high normal range).

We examined several compounds for possible interference with plasma NMN and MN determinations, including norepinephrine, epinephrine, isoproterenol, phenolamine, propranolol, indomethacin, yohimbine, desipramine, and acetaminophen. Compounds that were detectable after direct injection were then examined for interference after extraction from plasma.

Statistics. Differences between recoveries of metanephrines and internal standards were assessed by analysis of variance with post hoc tests carried out by Scheffe's method (13).

Results

Chromatography. The endogenous metanephrines (NMN, MN, and MHPEA) and the exogenous internal standards (FNMM, HMB, and EHPEA) were separated from each other and were all eluted from the column within 30 min after injection (Figure 2, upper panel). After extraction from plasma, NMN and MN were usually the most prominent endogenous compounds detected (Figure 2, middle and lower panels). Two other unknown compounds were always present on chromatograms of extracted plasma: one compound cochromatographed with the FNMM internal standard, and another eluted immediately after the EHPEA internal standard. The O-methylated metabolite of dopamine, MHPEA, was not detected in plasma samples from normal volunteers or hospital inpatients, but was occasionally detected in samples from patients with pheochromocytoma.

Sensitivity. Maximum detector responses to injection of both NMN and MN standards were attained at a reducing potential of $-0.43$ V at the third electrode. On this basis, and because of the instability of the detector output beyond $-0.40$ V, we chose a potential of $-0.39$ V for optimal detection of metanephrines. At this detecting potential, the electrochemical response varied linearly with the amounts of injected NMN or MN from 0.13 to 55 pmol.

The detector response to injection of MN standard was severely compromised when the pH of the mobile phase exceeded 3.5; at pH 4.0, the detector response was reduced by 55% and at pH 4.5 was reduced by 90%. The response for all other standards was unaffected. To optimize detection of MN, we therefore maintained the pH of the mobile phase between 3.2 and 3.4. Within this pH range and at the reducing potential stipulated above, the detection limit of the assay (signal-to-noise ratio of 3:1) was 25 fmol for NMN, 50 fmol for MN, and 120 fmol for MHPEA.

Analytical recovery. Recoveries of the three endogenous metanephrine standards and the three internal standards differed depending on whether the standards were added to water or plasma (Table 1). Recoveries of the standards varied greatly when extracted from water: the recovery of the HMB internal standard was less than that of NMN, but not of MN; the recovery of the EHPEA internal standard was greater than that of MN and HMB, but not of NMN. The recoveries of the standards extracted from plasma tended to be more
uniform: recoveries of NMN, MN, and the HMBA internal standard were similar; however, the recovery of the FNMN internal standard was significantly less and the recovery of the EHPEA internal standard was significantly more than recoveries of all other standards (all \( P < 0.01 \)). The relative recoveries of standards indicated that HMBA was the most appropriate internal standard for estimating plasma concentrations of NMN and MN.

Subsequent use of HMBA as an internal standard to correct for loss during extraction of 0.13 to 55 pmol of metanephrines added to 1-mL samples of plasma yielded highly linear relationships and close agreement between the amounts of NMN or MN added and the amounts recovered (Figure 3).

**Precision.** Intra-assay CVs were <5% for plasma concentrations of NMN and MN in the normal range (Table 2). Interassay CVs were greater, but were <11% for plasma NMN and MN concentrations in the high-normal range and <16.5% for concentrations in the low-normal range.

**Specificity.** Of the substances tested for chromatographic interference, only norepinephrine, epinephrine, isoproterenol, and acetaminophen were detected after direct injection (Table 3). Recoveries of norepinephrine, epinephrine, and isoproterenol from plasma were 0.6–0.8%. Because of these low recoveries and their different retention times relative to those of NMN and MN, the catecholamines were not a source of interference. The recovery of acetaminophen was even lower (0.06%) than for the catecholamines; however, acetaminophen was readily detected in plasma after oral administration of 500 mg of the drug (Figure 4). Because of this, and because of the similar retention times of acetaminophen and MN, acetaminophen is a potential source of interference with these measurements of NMN.

**Plasma concentrations.** Venous plasma concentrations of metanephrines in normal volunteers and hospital inpatients (without pheochromocytoma) were <0.73 nmol/L in all 32 subjects (Figure 5). In normal volunteers, plasma NMN concentrations were between 0.18 and 0.65 nmol/L (mean ± SD, 0.43 ± 0.15 nmol/L) and did not differ from values in hospital inpatients whose plasma concentrations of NMN were between 0.12 and 0.73 nmol/L (0.34 ± 0.16 nmol/L). Plasma MN concentrations in normal volunteers ranged between 0.10 and 0.63 nmol/L (0.25 ± 0.14 nmol/L) and also did not differ from concentrations in hospital inpatients, whose plasma MN concentrations were between 0.06 and 0.20 nmol/L (0.13 ± 0.04 nmol/L).

In patients with pheochromocytoma, the plasma concentrations of NMN and MN varied considerably, but in all 23 patients the NMN values (range 1–172 nmol/L) were above the range of values in normal volunteers.

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**Table 1. Recoveries of Metanephrines (NMN and MN) and Internal Standards (FNMN, HMBA, and EHPEA) Added to Water or Plasma**

<table>
<thead>
<tr>
<th></th>
<th>% recovery, mean ± SE</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>NMN</td>
</tr>
<tr>
<td>Water</td>
<td>12</td>
</tr>
<tr>
<td>Plasma</td>
<td>32</td>
</tr>
</tbody>
</table>

\(^a\) Significantly \( P < 0.01 \) different from extraction of \(^b\) NMN, \(^c\) MN, \(^d\) FNMN, and \(^e\) HMBA.
Table 2. Inter- and Intra-Assay Coefficients of Variation

<table>
<thead>
<tr>
<th></th>
<th>Low normal*</th>
<th>High normalb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-assay CV, % (n = 5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NMN</td>
<td>4.4</td>
<td>3.9</td>
</tr>
<tr>
<td>MN</td>
<td>2.6</td>
<td>3.9</td>
</tr>
<tr>
<td>Interassay CV, % (n = 20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NMN</td>
<td>14.4</td>
<td>10.1</td>
</tr>
<tr>
<td>MN</td>
<td>16.3</td>
<td>8.4</td>
</tr>
</tbody>
</table>

* NMN, 0.34 nmol/L; MN, 0.22 nmol/L.

b NMN, 0.75 nmol/L; MN, 0.44 nmol/L.

Intra-assay CVs were determined by repeated measurements performed during a single extraction and assay run carried out over one day. Interassay CVs were assessed by repeated measurements performed during extraction and assay runs carried out on separate days.

Table 3. Interference by Selected Drugs and Compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time,*</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMN</td>
<td>1.00</td>
<td>63</td>
</tr>
<tr>
<td>MN</td>
<td>1.42</td>
<td>63</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>0.57</td>
<td>0.8</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>0.73</td>
<td>0.6</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>2.37</td>
<td>0.6</td>
</tr>
<tr>
<td>Acetaminophenb</td>
<td>1.05</td>
<td>0.06</td>
</tr>
<tr>
<td>Phentolamine</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>Yohimbine</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>Desipramine</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>Propranolol</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>ND</td>
<td>–</td>
</tr>
</tbody>
</table>

* Relative to NMN.

b Interfering compound.

ND, not detectable.

Discussion

The few reports that describe methods for measuring plasma metanephrines yield inconsistent results. Using a radioenzymatic method, DeQuattro and colleagues (8, 14) reported plasma concentrations of free NMN of 0.33–0.66 nmol/L in normal volunteers, whereas others have reported plasma NMN concentrations of 1.8 to 3.1 nmol/L (9) or >21 nmol/L (10) by liquid-chromatographic procedures. Plasma concentrations of free MN were estimated to be between 1.9 and 2.4 nmol/L by one method (9) and undetectable by the other (10). Because the radioenzymatic assay depends on the enzymatic conversion of NMN to tritium-labeled MN, plasma MN could not be measured by this method.

Recently, a liquid-chromatographic method was described for simultaneous measurements of plasma concentrations of NMN and MN (11). Plasma concentrations of free NMN estimated by this method (~0.44 nmol/L) were similar to those reported by the radioenzymatic method (8, 14). Plasma concentrations of MN were one-third to one-half those of NMN. Plasma concentrations of total (free and conjugated) NMN or MN were 10-fold greater than for free metanephrines. Although the sensitivity of the assay was adequate for measurement of total metanephrines, the concentrations of free metanephrines were close to the detection limits of the assay (0.12 pmol). The two- to fourfold greater sensitivity of the present method over that described by Pagliari et al. (11) has enabled accurate and reliable measurements of free NMN and MN in 1-mL samples of plasma. Plasma concentrations of free NMN and MN in normal volunteers and hospital inpatients were similar to those reported by Pagliari et al. (11).

Improved sensitivity of the present method for detecting the low concentrations of free metanephrines in plasma was possible by coupling optimized detection and chromatographic conditions with the efficacy of the extraction procedure to purify and concentrate plasma NMN and MN. The efficacy of the extraction procedure is illustrated by the observation that NMN and MN and inpatients (Figure 5). In contrast, plasma MN concentrations ranged from 0.10 to 382 nmol/L, and in only nine of the patients with pheochromocytoma were the plasma MN concentrations above the range of values in normal volunteers and inpatients.

Fig. 4. Chromatograms of extracted samples of plasma obtained from a normal volunteer before (upper) and 3 h after (lower) oral ingestion of 500 mg of acetaminophen. A peak with a retention time identical to the retention time of the acetaminophen standard was present in all samples taken from subjects after ingestion of acetaminophen.
represent the major endogenous compounds detectable after injection of plasma extracts (Figure 2). Optimum potential settings of the series of triple electrodes included the use of a reducing potential rather than the more conventional oxidizing potential. This minimized interference from contaminating substances and thereby improved detection of metanephrines, which are usually present in the reduced state, at the reducing electrode. The observation that the detector response to MN, but not to the other metanephrines, depends on mobile phase pH illustrates the importance of considering this variable for adequate detection of MN.

To correct plasma NMN and MN values for loss during extraction, we examined the use of three possible internal standards. Although these compounds had chemical structures similar to metanephrines (Figure 1), they behaved differently from the metanephrines upon extraction, depending on whether they were extracted from water or plasma (Table 1). This behavior influences the choice of the most appropriate internal standard for measuring metanephrines in different fluid matrices. For extraction from plasma, recoveries of the commercially available HMBA best approximated those of the metanephrines; therefore, we consider HMBA the most appropriate internal standard for this fluid matrix.

Using HMBA to correct plasma concentrations of NMN and MN for loss during extraction yielded accurate measurements for samples to which known amounts of metanephrines had been added over a wide range of concentrations (Figure 3). The precision of these measurements was further validated by within-assay CVs of <5% for concentrations of metanephrines within the normal range (Table 2).

The extraction procedure used to purify plasma metanephrines, combined with the series electrode array, considerably enhanced the selectivity of the assay and largely eliminated interference from endogenous substances and drugs (Table 3). A notable exception to this was interference from acetaminophen, which, although not efficiently extracted, was present in plasma after oral ingestion at concentrations sufficient to be detected by the assay (Figure 4). The similar retention time of this drug with that of NMN indicates the need to restrict the use of this frequently prescribed medication in subjects whose plasma NMN concentrations are to be determined by the method.

As metabolites of catecholamines, the metanephrines may be less prone to rapid fluctuation than the parent compounds themselves. Thus, determination of plasma metanephrines might prove useful for the diagnosis of patients thought to have a pheochromocytoma. In support of this contention, the plasma concentrations of NMN were above normal in all 23 pheochromocytoma patients (Figure 5). In contrast, the plasma MN values in pheochromocytoma patients clearly overlapped those of the normal subjects. Normal plasma concentrations of MN in most of the pheochromocytoma patients are consistent with normal plasma concentrations of epinephrine frequently found in these patients (15). In the pheochromocytoma patients we studied, only 26% had above-normal plasma concentrations of epinephrine.

The consistently increased plasma concentrations of NMN in patients with pheochromocytoma agreed with the increased plasma concentrations of NMN reported by Foti et al. (14) in a smaller group of pheochromocytoma patients. In that study, plasma concentrations of NMN were determined by a radioenzymatic assay that did not allow simultaneous measurement of plasma MN. Because a minority of patients with pheochromocytoma may present with normal or near-normal plasma concentrations of norepinephrine and increased plasma concentrations of epinephrine (15), simultaneous measurements of MN and NMN may confer some advantage to the diagnosis of pheochromocytoma over the use of plasma NMN alone. However, the diagnostic value of determination of plasma metanephrines, in particular the positive and negative predictive value of a test result, remains to be established. Study of a control group of hypertensive subjects who do not have a pheochromocytoma is necessary for this purpose.

The described assay procedure for the simultaneous measurement of plasma NMN and MN is specific, sensitive, reliable, and accurate, and may be useful for the study of the release and extraneuronal metabolism of catecholamines. This may be particularly relevant in...
the development of new therapeutic agents for blocking catechol-O-methyltransferase or extraneuronal uptake, where measurements of metanephrines may serve as markers of potential drug effects on extraneuronal uptake and metabolism of catecholamines. The method may also be useful for the diagnosis of pheochromocytoma.

References
6. Westerink BHC. Determination of normetanephrine, 3,4-dihydroxyphe-nyleneglycol (free and total), and 3-methoxy-4-hydroxyphe-nyleneglycol (free and total) in rat brain by high-performance liquid chromatography with electrochemical detection and effects of drugs on regional concentrations. J Neurochem 1984;42:384-42.