Quantification of Unconjugated Metanephrines in Human Plasma without Interference by Acetaminophen

MICHAEL RODEN,¹ WOLFGANG RAFFESEBRG,¹ WOLFGANG RABER,¹ ELISABETH BERNROIDER,¹ BRUNO NIEDERLE,² WERNER WALDHÄUSL,¹ and SLOBODAN GASIC¹

Background: Pheochromocytoma is a rare cause of hypertension resulting from increased catecholamine secretion. We aimed to develop a method to measure unconjugated plasma normetanephrine (NMN) and metanephrine (MN) without interference from acetaminophen, a widely prescribed drug for headaches.

Methods: Plasma samples were obtained from 48 subjects (23 males, 25 females; mean age, 49 ± 14 years; hypertension, n = 37) under resting conditions. Following extraction on solid-phase cation-exchange columns, unconjugated metanephrines were analyzed by HPLC with electrochemical detection and with 4-hydroxy-3-methoxybenzylamine as an internal standard. Catecholamines were measured by HPLC.

Results: The assays were linear up to 2000 pg for NMN and for MN. Intraassay imprecisions (CVs) were 4.7% for NMN and 7.0% for MN, and the interassay CV was 12% for both NMN and MN. The limit of detection was 11 fmol for NMN and 17 fmol for MN. Ingestion of acetaminophen or its addition to plasma did not interfere with the MN peaks. Plasma NMN and MN were positively correlated (r = 0.52 and 0.49, respectively; P <0.01 for both) with the respective catecholamines. Plasma NMN (r = 0.27; P = 0.02) but not MN positively correlated with age, whereas only plasma catecholamines (and not metanephrines) were positively correlated (P <0.05) with diastolic blood pressure.

Conclusions: This sensitive MN assay is not affected by simultaneous acetaminophen medication, and reveals a correlation of metanephrines with plasma and urinary catecholamines and age but not with blood pressure.

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Pheochromocytomas are tumors of chromaffin cells most frequently originating from the adrenal medulla and represent a rare cause of secondary hypertension attributable to excessive production of norepinephrine (NE)³ and/or epinephrine (E) (1). Because almost every other afflicted patient has no or only episodic hypertension and not all suffer from classic clinical symptoms (1), highly sensitive tests are mandatory for the diagnosis of the disease (2).

Standard biochemical testing, such as the measurement of urinary vanillylmandelic acid and catecholamine excretion or of plasma catecholamine concentrations, can be influenced by external factors such as posture (3), stress (3, 4), or difficulty in obtaining complete 24-h urine collections (2, 5). Quantification of plasma concentrations of the metanephrines normetanephrine (NMN) and metanephrine (MN), the O-methylated extraneuronal metabolites of NE and E, respectively, may overcome these limitations. Measuring total, i.e., both unconjugated and conjugated metanephrines, increases the sensitivity (6) but is less accurate than free, i.e., unconjugated metanephrines for the detection of pheochromocytoma (7). This could well relate to the preparation procedures, which require acid hydrolysis or enzymatic deconjugation by sulfatase before measurement of total metanephrines. Unconjugated plasma NMN and MN also identify pheochromocytoma in subjects with familial predisposition for the tumor (8) and patients with adrenomedullary hypofunction (9). These studies used HPLC with electrochem-

¹ Division of Endocrinology and Metabolism, Department of Medicine III, and ² Department of Surgery, University of Vienna Medical School, A-1090 Vienna, Austria.

*Address correspondence to this author at: Division of Endocrinology and Metabolism, Department of Internal Medicine III, University of Vienna Medical School, Währinger Gürtel 18-20, A-1090 Vienna, Austria. Fax 43-1-40400-7790; e-mail michael.roden@akh-wien.ac.at.

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3 Nonstandard abbreviations: NE, norepinephrine; E, epinephrine; NMN, normetanephrine; MN, metanephrine; and HMBA, 4-hydroxy-3-methoxybenzylamine.
clinical detection to quantify plasma metanephrines (10–12). The method of Lenders et al. (11) provides a two- to fourfold greater sensitivity, but still suffers from interference by aceterminophen, a frequently prescribed analgesic drug that may be also used to treat headache of hypertensive patients.

The present study describes a simple HPLC method for the determination of unconjugated NMN and MN in human plasma that (a) has been shown to offer high diagnostic accuracy compared with plasma catecholamines for the detection of pheochromocytoma (3) and (b) is not affected by simultaneous aceterminophen medication.

Materials and Methods

Subjects

Patients were recruited from the Hypertension and Diabetes Outpatient Services of the Department of Internal Medicine III and from inpatients of the Department of Surgery at the University of Vienna Medical School. The anthropometric data of patients with or without hypertension (defined by systolic blood pressure >135 mmHg or diastolic blood pressure >85 mmHg) are shown in Table 1. Because kidney failure may affect the plasma metanephrine concentration (13), only patients with serum creatinine within the reference interval were included. None was taking aceterminophen, which was previously shown to interfere with the plasma NMN assay (5).

The study was performed in accordance with the current revision of the Helsinki Declaration of 1975 and the guidelines for Good Clinical Practice.

Protocols

Blood sampling was performed on patients in sitting or lying position, which do not affect unconjugated metanephrines (3). Heart rate and blood pressure were recorded simultaneously. Venous blood (5 mL) was collected in chilled heparin-containing tubes through an intravenous cannula in the forearm. The blood samples were immediately placed in ice-water and centrifuged (4 °C at 1400g) within 15 min; the supernatant was stored at −80 °C until assayed. In addition, three 24-h urine samples (of which the highest concentrations are given) for the determination of NE and E were obtained within 2–4 weeks before or after blood sampling.

To examine the reported interference of aceterminophen with the determination of plasma NMN, five healthy volunteers were admitted to the Clinical Research Unit. After an overnight fast, a plastic catheter was introduced into a forearm vein, and blood was drawn as described above after 15 min (baseline). The subjects then ingested aceterminophen at an antipyretic effective dose of 500 mg (Moxalan®; Merckle), and blood samples were collected 60 and 120 min later.

Determination of Plasma Unconjugated Metanephrines

The assay described here was recently used to determine plasma concentrations of unconjugated MN and NMN in patients with adrenal tumors before and after surgery (3). The assay represents the modification and optimization of a previously described method (11).

Sample extraction. All reagents, water, and methanol used for the extraction procedures were HPLC grade. The ion-exchange matrix of the solid-phase cation-exchange column (ICT ISOLUTE; 500 mg, 6 mL) was activated by washing three times with 5 mL of ammoniac methanol (7.5 mL of concentrated ammonia, 67.5 mL of water, 25 mL of methanol), once with 2.5 mL of potassium hydroxide in methanol (1 g/L), and once with 2.5 mL of water.

For adsorption of metanephrines, 1 mL of plasma containing the internal standard, 4-hydroxy-3-methoxy-benzylamine (HMBA; 1000 pg in 100 μL of 0.1 mol/L acetic acid), and 400 μL of 0.1 mol/L acetic acid were dissolved with water (final volume, 6 mL). Pooled plasma (control plasma) and pooled plasma supplemented with 1000 pg each of NMN and MN were treated identically. Columns were washed once with 6 mL of 10 mmol/L acetic acid–mehanol (9:1 by volume), once with 5 mL of water, once with 5 mL of 10 mmol/L ammonium phosphate (pH 7.8), and twice with 5 mL of water. The metanephrines were eluted with one 3.5-mL volume of ammoniac methanol, and the eluate was concentrated by vacuum centrifugation and lyophilized. Samples were stored at −80 °C until further use.

Table 1. Anthropometric characteristics and laboratory data in the total group and in selected subgroups.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total</th>
<th>Male</th>
<th>Female</th>
<th>Normotensive</th>
<th>Hypertensive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Total</td>
<td>48</td>
<td>23</td>
<td>25</td>
<td>11</td>
<td>37</td>
</tr>
<tr>
<td>Systolic</td>
<td>49 ± 14</td>
<td>49 ± 16</td>
<td>50 ± 12</td>
<td>51 ± 19</td>
<td>48 ± 13</td>
</tr>
<tr>
<td>Diastolic</td>
<td>148 ± 27</td>
<td>145 ± 21</td>
<td>151 ± 31</td>
<td>118 ± 14</td>
<td>157 ± 22</td>
</tr>
<tr>
<td>Plasma metanephrines, pmol/L</td>
<td></td>
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<tr>
<td>NMN</td>
<td>296 ± 134</td>
<td>361 ± 138</td>
<td>237 ± 101</td>
<td>267 ± 157</td>
<td>302 ± 130</td>
</tr>
<tr>
<td>MN</td>
<td>108 ± 60</td>
<td>131 ± 69</td>
<td>86 ± 41</td>
<td>108 ± 83</td>
<td>108 ± 52</td>
</tr>
<tr>
<td>NE</td>
<td>1606 ± 691</td>
<td>1703 ± 716</td>
<td>1525 ± 679</td>
<td>1104 ± 658</td>
<td>1704 ± 680</td>
</tr>
<tr>
<td>E</td>
<td>189 ± 128</td>
<td>206 ± 101</td>
<td>175 ± 150</td>
<td>132 ± 82</td>
<td>201 ± 139</td>
</tr>
<tr>
<td>Urinary catecholamines, nmol/24 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>NE</td>
<td>397 ± 271</td>
<td>484 ± 310</td>
<td>299 ± 181</td>
<td>282 ± 145</td>
<td>414 ± 288</td>
</tr>
<tr>
<td>E</td>
<td>45 ± 35</td>
<td>56 ± 41</td>
<td>33 ± 22</td>
<td>41 ± 25</td>
<td>46 ± 37</td>
</tr>
</tbody>
</table>

*Data are presented as means ± SD.
Chromatography. The mobile phase consisted of 0.85 mmol/L octane sulfonate, 0.16 mmol/L EDTA, 0.55 mol/L sodium dihydrogen phosphate, and 45 mL/L acetonitrile; the pH was adjusted with 85% o-phosphoric acid to 3.3 (all reagents were HPLC grade). Following extraction, the lyophilized material was reconstituted in 125 mL of mobile phase, and 50 mL was injected (Rheodyne 7125 Injector). The mobile phase was pumped through the chromatographic system at a flow rate of 1.25 mL/min. Liquid chromatography was performed on a HPLC column (Waters-Spherisorb; S5 ODS2, 4.6 X 250 mm; 5-μm particle size; Bischoff Chromatography) using the ESA 580 HPLC with two additional pulse dampers (Environmental Sciences Associates). For quantification of MN and NMN, we used the Coulochem II electrochemical detector equipped with a Model 5021 conditioning cell and Model 5014A microdialysis cell (Environmental Sciences Associates). Potentials for electrochemical detection were as follows: +0.4 V for the conditioning cell; +0.1 V for the first electrode of the microdialysis cell; −0.065 V for the second electrode of the microdialysis cell. The signal of the latter electrode was used for analysis. Data were transferred and analyzed using a software package (Perkin-Elmer-Nelson 900 integration interface and integration software; Perkin-Elmer Nelson Systems) on an IBM PS2 personal computer.

PLASMA CATECHOLAMINES
Plasma catecholamines were measured by reversed-phase HPLC using plasma catecholamine extraction tubes (Environmental Sciences Associates) for the isolation procedure (14). Inter- and intraassay CVs were <5% for both compounds (3).

URINE CATECHOLAMINES
Urine samples were collected in plastic flasks containing 10 mL of 8 mol/L HCl. After extraction by ion-exchange columns and separation by HPLC, catecholamines were measured by electrochemical detection (Pharmacia LKB) using reagent sets from Chromsystem (3).

ASSAY VALIDATION
Intraassay CVs were assessed from multiple (n = 20) measurements of NMN and MN in pooled plasma (target concentrations, ~700 pmol/L for NMN and ~200 pmol/L for MN) within one extraction procedure. Interassay CVs were calculated from repeated measurements of NMN and MN.

Fig. 1. Typical chromatogram of a plasma sample obtained from a healthy male subject under resting conditions (A) and the same sample with NMN and MN added (B). (A), HMBA was used as an internal standard (IS). Concentrations of NMN, MN, and HMBA, 275, 107, and 65 ppmol/L, respectively. (B), for identification and quantification of metanephrines, 1000 pg each of NMN and MN was added to the same plasma sample (1 mL) before extraction. Final NMN and MN concentrations, 5748 and 5191 pmol/L, respectively.

Fig. 2. Linearity of the assays for NMN (A) and MN (B). Defined amounts of NMN and MN were added to triplets of pooled plasma before standard analysis. The observed concentrations were plotted against added concentrations of the respective metanephrines, and linear regressions were calculated.
and MN in a different pooled plasma (target concentrations, 400 pmol/L for NMN and 200 pmol/L for MN) after extraction on different days. The limit of detection of the assay was assessed from the peak heights of NMN or MN equaling three times the basal oscillation (signal-to-noise ratio, 3:1).

**Statistical Analysis**

All data are presented as means ± SD unless stated otherwise. Correlation between variables was described by the Pearson rank correlation. \( P < 0.05 \) was considered statistically significant.

**Results**

**Performance of the Metanephrine Assay**

A typical chromatogram of a blood sample obtained from a healthy man under resting conditions is presented in Fig. 1A. For identification and quantification of metanephrines, 1000 pg each of NMN and MN was added to the same plasma sample (1 mL) before extraction (Fig. 1B). Linearity of the assays for MN and NMN was observed after addition of defined amounts (0, 100, 200, 500, 1000, and 2000 pg) of MN (0, 547, 1095, 2737, 5473, and 10 947 fmol) and NMN (0, 508, 1017, 2542, 5084, and 10 168 fmol), which were added to triplets of pooled plasma before standard analysis (Fig. 2).

Minor differences in the recoveries of NMN and MN (90–105%) compared with the internal standard HMBA between different series of extraction were corrected by use of control plasma and supplemented control plasma.

Intraassay CVs were 4.7% for NMN (plasma NMN, 671 ± 32 pmol/L) and 7.0% for MN (plasma MN, 153 ± 11 pmol/L). The interassay CV was 12% for both NMN (plasma NMN, 437 ± 54 pmol/L) and MN (plasma MN, 210 ± 25 pmol/L). The limits of detection were 11 fmol for NMN and 17 fmol for MN. The limits of quantification in plasma extracts were 66 pmol/L for NMN and 44 pmol/L for MN.

For studies on the interference of acetaminophen with plasma NMN, one tablet of Mexalen containing 500 mg of acetaminophen was suspended in 100 mL of water–methanol (4:1 by volume). Aliquots of the supernatant (100 μL each) were added as an external acetaminophen standard to aliquots of baseline plasma sample before extraction and analyzed by HPLC (Fig. 3A). In addition, blood samples were drawn from healthy volunteers after ingestion of 500 mg of Mexalen. Under our experimental conditions, no interference of the drug with the NMN and MN peaks was found after 1 and 2 h. The relative retention factors for NMN and MN were 0.67 and 1.21 compared with acetaminophen (relative retention factor = 1.00) as shown in a typical chromatogram (Fig. 3B).

Chromatograms of one typical patient with histologically confirmed pheochromocytoma showed increased...
concentrations of NMN (20 500 pmol/L) and MN (12 200 pmol/L; Fig. 4A) and the expected decrease to reference values (361 and 71 pmol/L, respectively) after surgical removal of the tumor (Fig. 4B).

**PATIENT DATA**

Anthropometric data and concentrations of catecholamines and metanephrines of the total study population as well as of defined subgroups are summarized in Table 1. Thirty-seven (77%) of the subjects were hypertensive. Serum creatinine was slightly but significantly higher \( (P < 0.05) \) in male than in female subjects. Plasma NMN concentrations were higher \( (P < 0.01) \) in male than in female subjects.

Plasma NMN and MN were positively correlated with the respective plasma \( (r = 0.52 \text{ and } 0.49; P < 0.01 \text{ for both}) \) and urinary catecholamines, NE \( (r = 0.31; P = 0.04) \) and E \( (r = 0.38; P = 0.03; \text{Fig. } 5) \). Plasma NMN was weakly but significantly \( (P = 0.02) \) related to age, which did not hold true for plasma MN and catecholamines (Fig. 6). Plasma NE was positively correlated with systolic \( (r = 0.19; P = 0.04) \) and diastolic \( (r = 0.20; P = 0.04) \) blood pressure, whereas plasma E was correlated with diastolic \( (r = 0.30; P = 0.03) \) but not with systolic blood pressure \( (r = 0.15; \text{Fig. } 6) \).

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Fig. 5. Correlation of plasma metanephrines with plasma and urinary catecholamines in the patients described in Table 1. Plasma NMN is plotted against plasma \( (A) \) and urinary NE \( (C) \). Plasma MN is plotted against plasma \( (B) \) and urinary NE \( (D) \) and correlated with the Pearson rank correlation test.
Both plasma NMN and MN were unrelated to systolic or diastolic blood pressure.

**Discussion**

The described assay for the determination of unconjugated plasma metanephrines is based on the method described by Lenders et al. (11). The latter method was modified by the use of (a) different solid-phase extraction columns, (b) a different chromatographic system (column material, mobile phase), and (c) a coulometric detector with a different analytical cell, which allowed improved selective setting of oxidizing and reducing potentials.

Both methods allow detection of pheochromocytomas with higher sensitivity and specificity compared with the measurement of plasma catecholamines (3, 7, 8). Baseline concentrations of NMN and MN under resting conditions were in good agreement with the previous method (11) and some radioenzymatic techniques (15, 16), but different from other methods (10, 17, 18). The greater sensitivities of both newer methods and the resulting lower limits of detection most likely explain these differences from previous assays. The intra- and interassay CVs for NMN were comparable to those reported by Lenders et al. (11). The mean values for our control subjects were slightly higher than the values reported in that study (11), but were very similar to the values obtained in other studies by the same group (4, 7–9, 12) as well as those in our recent report (3).

Although otherwise comparable, the major advantage of the present assay compared with the other established HPLC method (11) resides in its ability to clearly separate the NMN peak from that of the analgesic and antipyretic drug acetaminophen. Because bilateral diffuse headache is typical for essential hypertension but also belongs to the classical symptom triad of pheochromocytoma (1), patients at risk of pheochromocytoma can be expected to use acetaminophen (as Tylenol® in the US or as Mexalen in Europe). Thus, application of the present method for the quantification of plasma NMN does not require patients to stop taking acetaminophen. Minor differences in the sample extraction procedures, particularly the binding pH of the sample and the cation-exchange column, which are also critical for the purification of the metanephrines (19), could have led to lower recoveries of acetaminophen and/or its metabolites. Slightly different chromatographic conditions, such as column material and mobile phase, gave retention factors for both NMN and MN relative to acetaminophen that enabled clear separation of the peaks of these compounds.

The data demonstrate that unconjugated plasma metanephrines not only correlate with the respective plasma concentrations of their parent compounds, as shown previously (4), but also with urinary excretion of the corresponding catecholamines. Nevertheless, NMN and MN are less sensitive to stimulation by insulin and glucagon (12), a change to upright posture (3), mental challenge (4), and intraoperative stress (3). These findings along with the good correlation of tumor size and volume with plasma metanephrine concentrations, which most likely results from intratumoral metabolism of catecholamines, are considered to explain the improved diagnostic efficacy for the detection of pheochromocytomas (3, 12).

With regard to their proposed role as tumor markers, it was of interest to evaluate plasma NMN and MN concentrations in a control population. The positive correlation between age and plasma NMN but not MN is in agreement with previous results obtained in a slightly younger population [mean age, ~40 years vs ~50 years (4)]. Such correlation was not detectable when total plasma meta-
nephrines were measured, which could be attributable to the markedly lower intraassay (≤7.0% each) and interassay imprecision (≤12.5% each) of that HPLC method (19). The positive relationship between plasma catecholamines and systolic as well as diastolic blood pressure is in agreement with sympathetic regulation of blood pressure (20).

In conclusion, this sensitive metanephrine assay is not affected by simultaneous acetaminophen medication and reveals a correlation of metanephrines with plasma and urinary catecholamines and age but not with blood pressure.

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