

Plasma Free Metanephrine Measurement Using Automated Online Solid-Phase Extraction HPLC–Tandem Mass Spectrometry

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Background: Quantification of plasma free metanephrine (MN) and normetanephrine (NMN) is considered to be the most accurate test for the clinical chemical diagnosis of pheochromocytoma and follow-up of pheochromocytoma patients. Current methods involve laborious, time-consuming, offline sample preparation, coupled with relatively nonspecific detection. Our aim was to develop a rapid, sensitive, and highly selective automated method for plasma free MNs in the nanomole per liter range.

Methods: We used online solid-phase extraction coupled with HPLC-tandem mass spectrometric detection (XLC-MS/MS). Fifty microliters plasma equivalent was prepurified by automated online solid-phase extraction, using weak cation exchange cartridges. Chromatographic separation of the analytes and deuterated analogs was achieved by hydrophilic interaction chromatography. Mass spectrometric detection was performed in the multiple reaction monitoring mode using a quadrupole tandem mass spectrometer in positive electrospray ionization mode.

Results: Total run-time including sample cleanup was 8 min. Intra- and interassay analytical variation (CV)

varied from 2.0% to 4.7% and 1.6% to 13.5%, respectively, whereas biological intra- and interday variation ranged from 9.4% to 45.0% and 8.4% to 23.2%. Linearity in the 0 to 20 nmol/L calibration range was excellent ($R^2 > 0.99$). For all compounds, recoveries ranged from 74.5% to 99.6%, and detection limits were <0.10 nmol/L. Reference intervals for 120 healthy adults were 0.07 to 0.33 nmol/L (MN), 0.23 to 1.07 nmol/L (NMN), and <0.17 nmol/L (3-methoxytyramine).

Conclusions: This automated high-throughput XLC-MS/MS method for the measurement of plasma free MNs is precise and linear, with short analysis time and low variable costs. The method is attractive for routine diagnosis of pheochromocytoma because of its high analytical sensitivity, the analytical power of MS/MS, and the high diagnostic accuracy of free MNs.

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A characteristic of pheochromocytomas is the excessive secretion of catecholamines, which cause typical symptoms such as sustained or intermittent hypertension, sweating, tachycardia, and palpitations (1–4). The prevalence of pheochromocytoma in patients with hypertension is estimated to be 0.1% to 0.5%, with 20% to 75% of these cases remaining undetected before death (5, 6). Highly sensitive and specific biochemical tests are required for correct clinical chemical diagnosis, avoidance of false-negative results, and follow-up of patients.

The main metabolic routes of catecholamines are oxidative deamination by monoamine oxidase (EC 1.4.3.4) and *O*-methylation by catechol-*O*-methyltransferase (EC 2.1.1.6) or both. Metanephrines (MNs)⁶ are the products of

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⁶ Nonstandard abbreviations: MN, metanephrine; 3-MT, 3-methoxy-metanephrine; NMN, normetanephrine; NE, norepinephrine; ECD, electrochemical detection; SPE, solid-phase extraction; XLC, online SPE-liquid

enzymatic conversion of catecholamines by catechol-O-methyltransferase: 3-methoxymetanephrine (3-MT) from dopamine, normetanephrine (NMN) from norepinephrine (NE), and MN from epinephrine (1–4).

Traditional biochemical tests for the diagnosis of pheochromocytoma are based on the presence of several compounds in the catecholamine metabolic pathway. Some of these analyses have limitations with respect to analytical or diagnostic sensitivity or specificity and analysis time (1–4). Recent studies have highlighted the higher diagnostic accuracy of plasma free MN measurements over tests that quantify catecholamines. This difference is a consequence of constant leakage of catecholamines from vesicular stores (in tumor cells) into the cytoplasm, where they are continuously metabolized to MNs (7, 8). Although diagnostic specificity may be increased, false-positive results may still occur owing to interference from prescribed medications or diet (7, 9–11).

Because plasma MNs occur in low concentrations in a complex matrix and their chemical characteristics are not unique, developing an assay for these compounds is an analytical challenge. Currently, HPLC using electrochemical detection (ECD) is the method of choice (7, 12, 13). However, data interpretation may be complicated because of coeluting interference, and improvements can be made in reducing labor-intensive sample cleanup and total analysis time. Alternatively, rapid measurement of plasma free MN can be achieved by the use of immunoassays (14, 15); however, cross-reactivity and nonspecific binding may lead to erroneous results. In addition, the lack of an internal standard in such assays reduces confidence in reported values. GC-MS methods address some concerns regarding sensitivity and specificity. Nevertheless, sample preparation procedures for these methods involve derivatization and extraction, which are laborious and time-consuming. Given the limitations of existing analytical techniques, the need for an alternative, simplified method of analysis is clear.

An LC-MS/MS method using offline solid-phase extraction (SPE) (16) appears to be superior to GC-MS methodologies in terms of both sensitivity and sample throughput. However, considerable improvements could be made to the selectivity of the SPE procedure and sensitivity of the analysis. Furthermore, as sample preparation has been highlighted as a major contributor to the total analysis time observed in previous methods, automation of the extraction process and coupling it directly to the chromatographic system would be expected to lead to significant benefits in sample throughput and precision. This process is termed online SPE–liquid chromatography (XLC).

This study describes a high-throughput, sensitive, specific, and automated XLC-MS/MS method that enables

simultaneous extraction, concentration, separation, and mass-selective detection of plasma free MNs for the diagnosis of pheochromocytoma.

Materials and Methods

REAGENTS

We obtained HPLC-grade acetonitrile and methanol from Rathburn Chemicals; ammonium formate 99.995⁺% and disodium EDTA from Sigma-Aldrich; formic acid 98% to 100% ultrapure from BDH Laboratory Supplies; and sodium hydroxide (NaOH), hydrochloric acid, and sodium metabisulfite (Na₂S₂O₅) from Merck KGaA. Reagent-grade water, obtained from a Barnstead system, was used throughout the study procedure.

We purchased D,L-metanephrine-HCl, D,L-normetanephrine-HCl, and D,L-3-methoxytyramine-HCl from Sigma-Aldrich, the deuterated internal standards $\alpha,\alpha,\beta,\beta$ -d₃-metanephrine-HCl and $\alpha,\alpha,\beta,\beta$ -d₄-3-methoxytyramine-HCl from Cambridge Isotopes, and α,α,β -d₃-normetanephrine-HCl from Medical Isotopes.

STOCK SOLUTIONS AND SAMPLES

We prepared stock solutions in 0.1 mol/L HCl. Stock solutions were serially diluted and used to form calibrators and low, medium, and high quality-control samples in pooled plasma via enrichment. The concentration range of the calibrators was from physiological levels (0 to 1 nmol/L) to approximately 20 nmol/L for all analytes.

Plasma samples from patients with confirmed pheochromocytoma for whom these tests were ordered (as established by the routinely used GC-MS method for urinary fractionated MNs and pathology reports) came from our own hospital (University Medical Center, Groningen). We collected blood samples by venipuncture, with the patient in a sitting position, in 10-mL Vacutainer Tubes (Becton Dickinson) containing K₂EDTA solution as anticoagulant. After centrifugation, we transferred the plasma to glass tubes containing 5 mg Na₂S₂O₅ as a preservative, and samples were stored at –20 °C until analysis.

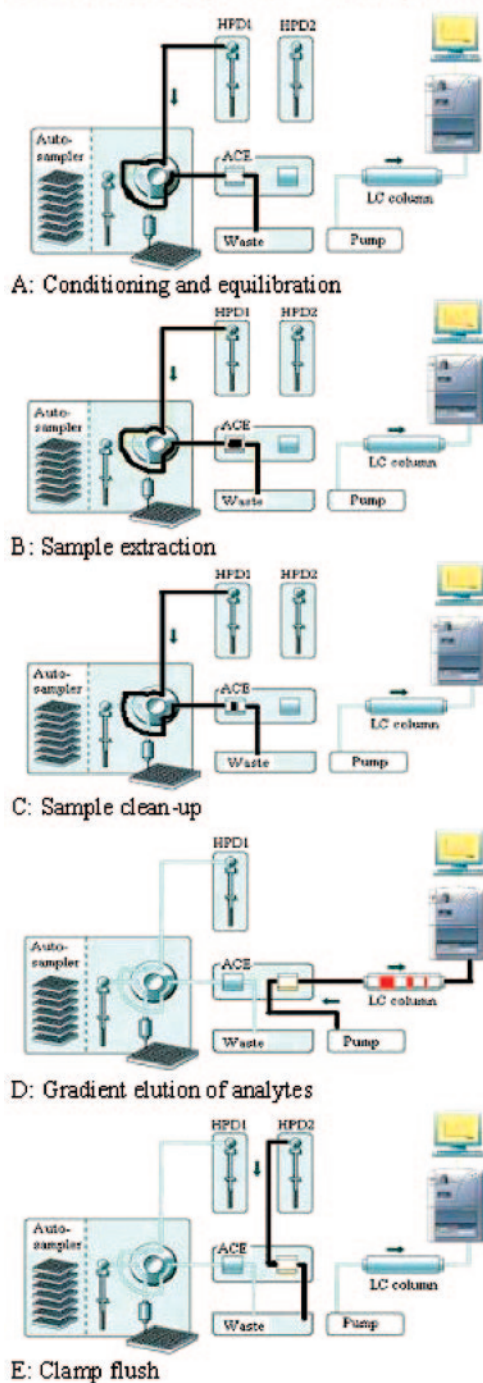
Before analysis, we mixed aliquots of plasma samples (500 μ L) with 100 μ L internal standard stock solution (4.95 nmol/L in diluted acetic acid) and diluted them with 400 μ L water. We placed sample vials in the autosampler, and 100 μ L of each sample (equivalent to 50 μ L of plasma) was injected. Required sample volume for automatic injection can be scaled down to 50 μ L by using microliter pickup injection mode.

ANALYSIS AND QUANTIFICATION

Instrumentation. We used a Spark Holland Symbiosis[®] online SPE system for all analysis. The system consists of a temperature-controlled autosampler (temperature maintained at 10 °C), a SPE controller unit (automated cartridge exchanger), a solvent delivery unit (2 high-pressure dispensers), and an HPLC pump, as shown in Fig. 1. The automated cartridge exchanger module con-

chromatography; MRM, multiple reaction monitoring; LOD, limit of detection; HILIC, hydrophilic interaction chromatography.

On-line SPE system MS system



A: Conditioning and equilibration

- | | |
|-------------------|------------------------------------|
| A1: New cartridge | From tray to left clamp |
| A2: Conditioning | Acetonitrile
(1 mL at 2 mL/min) |
| A3: Equilibration | Water (2 mL at 2 mL/min) |

B: Sample extraction

- | | |
|-----------------|--|
| B 1: Injection | Start loopfill with sample |
| B 2: Extraction | Sample loading using
water (500 μ L at 500 μ L/min) |

C: Sample clean-up

- | | |
|--------------------|--|
| C1: Wash cartridge | Water
(1 mL at 2.5 mL/min) |
| C2: Wash cartridge | Acetonitrile:Water (9:1)
(1 mL at 2.5 mL/min) |

D: Gradient elution of analytes

- | | |
|------------------------|---|
| D1: Transfer cartridge | From left to right clamp |
| D2: Elution | Elution of analytes from
cartridge to LC column
using LC gradient (1.0 min) |
| D3: New cartridge | From tray to left clamp |

E: Clamp flush

- | | |
|--------------------|---|
| E1: Clamp flush | Right clamp cartridge wash
with 10 mmol/L pH 3.0
ammoniumformate buffer
(1 mL at 1 mL/min) |
| E2: Clamp flush | Water (2 mL at 1 mL/min) |
| E3: Clamp flush | Acetonitrile
(2 mL at 2 mL/min) |
| E4: Move cartridge | From right clamp to tray |

Fig. 1. Schematic representation of the Symbiosis XLC-MS/MS.

(A), conditioning and activation of the SPE cartridge in the left clamp. (B), sample extraction after filling of the autosampler loop. (C), sample cleanup. (D), right clamp gradient elution of analytes with LC mobile phase, directly followed by chromatographic separation and mass spectrometric detection. (E), cartridge and clamp cleanup. HPD, high-pressure dispenser; LC, liquid chromatography.

tains 2 connectable 6-way valves and an SPE cartridge-exchange module. The high-pressure dispensers provide SPE cartridges with solvents for conditioning, equilibration, sample application, and cleanup. The integrated HPLC pump was a binary high-pressure gradient pump.

We used Oasis[®] weak cation exchange 10 by 1 mm SPE cartridges (Waters Corp.) for sample extraction and performed HPLC by use of an Atlantis HILIC Silica column (particle size 3 μ m, 2.1 mm internal diameter by 50 mm; Waters). Column temperature was controlled with a

Mistral Column Oven (Spark Holland). Detection was performed with a Quattro[®] Premier tandem mass spectrometer equipped with a Z Spray[®] ion source operated in positive electrospray ionization mode (Waters). All aspects of system operation and data acquisition were controlled using MassLynx v4.1 software with automated data processing using the QuanLynx Application Manager (Waters).

Online SPE. We performed online SPE following a similar method described by Kema et al. (17). The Symbiosis system was designed to proceed automatically through a series of programmable routines during which the SPE cartridge is loaded, washed, and eluted. The analytes were eluted directly on the analytical column, as is schematically shown in Fig. 1. In the 1st step, the SPE cartridge was automatically located in the left clamp for conditioning and equilibration (Fig. 1A). The sample was passed on to the extraction cartridge (Fig. 1B) using water as the loading solvent, and wash solvents were applied (Fig. 1C). The extraction cartridge was then automatically transferred to the right clamp for elution of the analytes directly on the analytical column by passing the chromatographic mobile phase through the cartridge for 2 min (Fig. 1D). After elution, chromatographic separation on the analytical column occurred and the right clamp, containing the cartridge, was flushed (Fig. 1E). Processing of subsequent plasma samples was carried out in parallel.

Liquid chromatography. The binary gradient system consisted of 100 mmol/L ammonium formate in water adjusted to pH 3.0 with formic acid (eluent A) and acetonitrile (eluent B). Gradient elution was performed according to the following elution program: 0 min, 5% A, 95% B; 6 min, 20% A, 80% B; 6–7 min, 20% A, 80% B; 7.5 min, 5% A, 95% B; reequilibration from 7.5 to 8 min with 5% A, 95% B. Gradients applied were linear; flow rate was 0.400 mL/min. Column temperature was kept at 20 °C.

Mass spectrometry. In positive ionization mode, MN, NMN, and 3-MT were protonated to produce ions at the form $[M+H]^+$. These ions are known to undergo a facile loss of water in the ion source, and the ion source conditions were optimized for these resulting ions of the form $[M+H-H_2O]^+$: MN, m/z 180; NMN, m/z 166, and 3-MT, m/z 151, as described by Lagerstedt et al. (16). On collision-induced dissociation, these precursor ions produced characteristic product ions of m/z 148, m/z 134, and m/z 119, respectively. We developed a multiple reaction monitoring (MRM) method using a dwell time of 40 ms and an interchannel delay of 10 ms. Recently, the use of additional MRM transitions for absolute confirmation of the presence of a compound in an analytical method has been proposed (e.g., EU directive 2002/657/EC). For this reason, we used mass transitions m/z 198→180 (MN), 184→134 (NMN), and 151→91 (3-MT) as qualifiers.

QUALITY CONTROL AND METHOD VALIDATION

Selectivity. We verified the identities of sample MN, NMN, and 3-MT peaks by analysis of the compound specific mass spectra after addition of calibrator (standard addition).

Detection limits. For plasma, we determined detection limits [limit of detection (LOD)] and quantification limits by injecting serially diluted samples containing MN, NMN, and 3-MT. LOD was defined as the injected amount that produced a signal-to-noise ratio of 3. Limit of quantification was defined as the injected amount that produced a signal-to-noise ratio of 10. We estimated the percentage of carryover between sequential analyses performed on new SPE cartridges by alternating injections of blanks and plasma samples with high concentrations of MNs.

Linearity and imprecision. We plotted the ratios of analyte peak area to internal standard peak area against MNs at 8 concentrations in the intervals 0.26 to 18.21 nmol/L for MN, 0.82 to 18.21 nmol/L for NMN, and 0.58 to 19.93 nmol/L for 3-MT. On 20 different days, we prepared and measured fresh calibration lines. The lines were calculated by use of Excel software and least-squares linear regression. We applied the CLSI EP-6P protocol (18) to test the linearity of the method. The dilutional linearity of the assay was performed in duplicate by serial dilution of enriched plasma samples with water.

We determined intra- and interassay variation by use of 3 pooled samples with MNs in low, medium, and high concentrations and obtained intraassay imprecision from 20 replicates measured in a single series and interassay imprecision from 20 different assays over a 3-week period.

Recovery. We estimated mean relative recoveries by the addition of MNs to plasma in low, medium, and high concentrations and measured recoveries in 6 replicates of these samples by using 2 cartridges placed in series.

Method comparison. Heparin-EGTA plasma samples measured previously, as described by Lenders et al. (13) using HPLC-ECD, were reanalyzed with the new XLC-MS/MS method. Samples were stored at –20 °C for 1 year. We applied Passing–Bablok regression analysis (using EP Evaluator, recommended by the CLSI) (19) for method comparison.

Stability. Samples with low, medium, and high concentrations of added MNs were measured in triplicate after different storage conditions. The 1st set was assayed immediately and served as reference point; other sets were stored at 10 °C (autosampler temperature) and 4 °C for 16, 24, and 48 h and 7 days and at room temperature for 24 h. The remaining samples were frozen at –20 °C,

and stability was investigated after 1 to 3 freeze-thaw cycles.

Biological variation, reference values, and patient samples. We determined biological intra- and interday variation by analyzing plasma obtained from 10 healthy individuals in sitting position (5 men, 5 women, age range 20 to 56 years, median age 35.0 years), at 5 times during 1 day (9:00 AM, 11:00 AM, 1:00 PM, 3:00 PM, and 5:00 PM) and on 5 consecutive days (at 9:00 AM), respectively. MN reference intervals were based on the analysis of 120 plasma samples derived from healthy individuals in a sitting position (63 men, 57 women, age range 36 to 81 years, median age 54.5), during the PREVEND study (20, 21). Both studies were approved by the medical ethics committee of our institution and conducted in accordance with the guidelines of the Declaration of Helsinki. All participants gave written informed consent. We calculated reference intervals by use of EP Evaluator (19).

We analyzed plasma samples from 10 patients with histologically proven pheochromocytoma to illustrate diagnostic value of the method.

Results

QUALITY CONTROL AND METHOD VALIDATION

Chromatography and selectivity. Total sample analysis time, including extraction, was 8 min. Complete chromatographic separation of the MNs is not necessary, whereas applying hydrophilic interaction chromatography (HILIC) chemistry reduces peak width and chromatographic time. Deuterated internal standards can be used, because the mass spectrometer monitors parent as well as daughter ions with high analytical specificity. Chromatograms obtained by XLC-MS/MS in MRM are shown in Fig. 2. We confirmed the identities of the compounds by the specific mass spectra. The mass chromatograms in Fig. 2, A–C, show the responses for MN, NMN, and 3-MT and the respective deuterated internal standards in a healthy individual, and Fig. 2, D–F, shows chromatograms with increased responses from a patient with histologically proven pheochromocytoma. Not all pheochromocytoma patients have increased plasma free 3-MT concentrations. Comparison of the chromatograms from the healthy individual with those of a pheochromocytoma patient reveals markedly increased MN (0.23 and 17.24 nmol/L, respectively; corresponding peak areas 2578 and 173 771) and NMN (1.03 and 18.03 nmol/L; peak areas 1820 and 15 253) concentrations in the patient sample. In healthy individuals, both MN and NMN are present in low but quantifiable amounts.

Detection limits. LOD was 0.01 nmol/L for MN, 0.02 nmol/L for NMN, and 0.04 nmol/L for 3-MT. Respective quantification limits (at a signal-to-noise ratio of 10) were 0.03, 0.05, and 0.06 nmol/L, with CVs of 10%, 13%, and 16%.

Cartridges could be reused several times, with carryover <0.1% observed between sequential analyses performed on reused SPE cartridges, by applying additional washing steps in the method.

Linearity and imprecision. Plasma calibration curves and control samples were run with every batch of patient samples. Linearity was excellent over the 0 to 20 nmol/L calibration range, with corresponding correlation coefficients (R^2) consistently >0.99 for all 3 compounds. Plasma calibration curves were reproducible between days, with $R^2 > 0.99$. Mean analytical intra- and interassay repeatability and reproducibility for enriched pooled plasma in low, medium, and high concentrations are shown in Table 1. Intraassay CV ($n = 20$) was 2.5% to 4.8% (MN), 5.1% to 6.2% (NMN), and 4.4% to 8.0% (3-MT). Interassay CV ($n = 20$) was 3.4% to 5.6% (MN), 4.2% to 7.1% (NMN), and 4.5% to 11.1% (3-MT). Reproducibility, recovery, and imprecision were measured in the same way with aqueous calibration curves, which gave comparable results (data not shown). Plasma samples with high MN concentrations that exceed the calibration range can be diluted up to 100 times.

Recovery. Recoveries ranged from 94.4% to 99.6% (MN), 74.5% to 99.1% (NMN), and 81.4% to 98.5% (3-MT) and are shown in Table 2 for low, medium, and high concentrations.

Method comparison. We compared the results obtained by XLC-MS/MS and HPLC-ECD methods for patient specimens routinely screened for pheochromocytoma. Passing–Bablok regression for HPLC-ECD and XLC-MS/MS results ($n = 50$) gave slopes of 1.19 (95% CI 1.02–1.62; R^2 0.9338) and 1.14 (1.06–1.25; 0.9675) and y -intercepts of 0.15 and 0.03 nmol/L for MN and NMN, respectively. No outliers were detected. 3-MT data could not be compared because with the HPLC-ECD method it is not possible to measure this marker in plasma.

Stability. MNs were stable in plasma stored up to 7 days at 10 or 4 °C. At room temperature, plasma MNs were stable up to 24 h. No changes in measured concentrations were observed in plasma that had been subjected to 1, 2, or 3 freeze-thaw cycles. Stability data ($n = 3$) are not shown.

Biological variation, reference values, and patient samples. Biological intra- and interday CVs ($n = 10$) were 9.4% and 8.4% (MN), 15% and 13% (NMN), and 45% and 23% (3-MT), as shown in Table 1.

The distribution of 120 reference values is right-shifted for all 3 MNs, as shown in Fig. 3. Therefore, we calculated reference intervals with EP evaluator in a transformed parametric manner according to CLSI C28-A2 (22). Reference intervals were 0.07 to 0.33 nmol/L (MN), 0.23 to

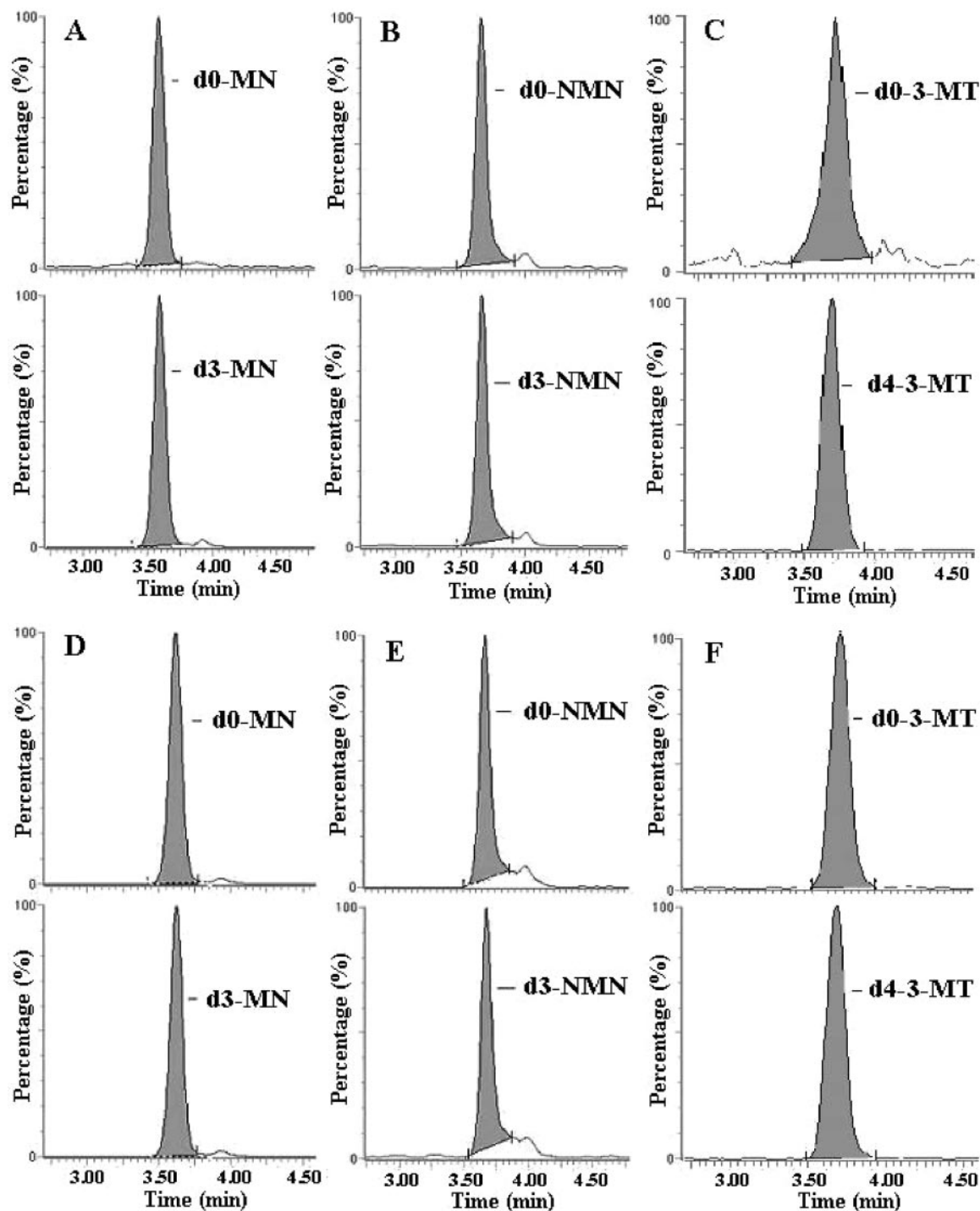


Fig. 2. Chromatograms of free plasma MN, NMN, and 3-MT (*d*0) and their deuterated internal standards (*d*3) obtained by XLC-MS/MS in MRM mode.

(A–C), MN (0.23 nmol/L; peak area *d*0: 2578, *d*3: 34499), NMN (1.03 nmol/L; *d*0: 1820, *d*3: 5544), and 3-MT (0.09 nmol/L; *d*0: 566, *d*3: 8754) in a healthy individual, respectively. (D–F), MN (17.24 nmol/L; peak area *d*0: 17377, *d*3: 33151), NMN (18.03 nmol/L; *d*0: 15253, *d*3: 2740), and 3-MT (1.07 nmol/L; *d*0: 8223, *d*4: 10298) in a patient with histologically proven pheochromocytoma. All signals are normalized to full scale for the highest peak in the window. Retention time is indicated in min. Parent–daughter transitions used for quantification were *m/z* 180→148 for MN, *m/z* 166→134 for NMN, and *m/z* 151→119 for 3-MT. *d*, deuterated.

1.07 nmol/L (NMN), and <0.17 nmol/L (3-MT). Plasma free MN concentrations of 10 patients with histologically proven pheochromocytoma, measured with XLC-MS/MS, are shown in Table 3.

Discussion

Measurement of *O*-methylated catecholamine metabolites (MNs) provides the best diagnostic accuracy for the biochemical diagnosis of pheochromocytoma (7, 10, 23).

Table 1. Intra- and interassay imprecision of the XLC-MS/MS method for plasma free MN, NMN, and 3-MT.^a

	Mean analytical variation (n = 20)						Mean biological variation (n = 10)					
	Intraassay			Interassay			Intraday			Interday		
	Mean, nmol/L	SD, nmol/L	CV, %	Mean, nmol/L	SD, nmol/L	CV, %	Mean, nmol/L	SD, nmol/L	CV, %	Mean, nmol/L	SD, nmol/L	CV, %
MN												
Low	0.53	0.01	2.50	0.23	0.01	5.55	0.22	0.02	9.43	0.26	0.02	8.41
Medium	1.66	0.08	4.82	1.41	0.08	5.41						
High	7.76	0.25	3.38	7.76	0.26	3.41						
NMN												
Low	0.59	0.03	5.37	0.59	0.04	7.08	0.47	0.07	15.18	0.50	0.07	13.44
Medium	1.65	0.10	6.18	1.68	0.11	6.34						
High	24.37	1.23	5.06	24.29	1.01	4.16						
MT												
Low	0.18	0.01	7.25	0.10	0.01	11.11	0.07	0.03	44.95	0.04	0.01	23.24
Medium	0.79	0.06	8.00	0.74	0.05	6.65						
High	3.03	0.13	4.42	3.04	0.13	4.25						

^a Analytical variation was calculated by measuring each sample 20 times per day (intraassay) and in 20 different assays (interassay).

Intra- and interday mean biological variation was calculated from results from 10 healthy individuals at 5 times during a day (9:00 AM, 11:00 AM, 1:00 PM, 3:00 PM, and 5:00 PM) and on 5 consecutive days at 9:00 AM.

Because most *O*-methylation takes place within the tumor cells that produce the catecholamines, the continuous production of MNs is independent of the highly variable sympathoadrenal catecholamine release in patients with pheochromocytoma. Consequently, concentrations of MNs are strongly correlated with tumor size (8). Furthermore, diagnostic specificity of MN assays is increased with regard to catecholamine methods, as the concentrations of free MNs are less affected by environmental factors, including stress and changes of posture during specimen collection (1, 2, 4, 8, 10), although it was recently recommended that MN blood samples should be collected in a supine position (24–26). Moreover, plasma MNs have higher stabilities than catecholamines, leading to simplified sample handling and storage (27, 28).

Measurement of plasma free MNs is an analytical challenge, because these compounds occur in low concentrations without unique chemical characteristics and are

difficult to isolate from the matrix. Several methods have been described for the determination of MNs, including HPLC with amperometric (13) or coulometric detection (29) and offline LC-MS/MS (16). These methods have certain drawbacks such as labor intensity and long analysis times. Automated sample preparation reduces analysis time and analytical variation caused by differences in manual sample pretreatment. Furthermore, online concentration is possible by increasing the ratio between the sample and elution volume. Basic technique principles have been described previously for other applications (17, 30). Main advantages of XLC-MS/MS are ease of handling, portability, and reduction of cost per sample, because of reduced sample preparation time, high throughput, cheaper cartridges, and reuse of cartridges. Maintenance of the online extraction system is comparable to that of conventional HPLC systems, whereas maintenance costs are approximately triplicate.

Because MN, NMN, and 3-MT contain the same functional charged amino group, a selective SPE process can be achieved using cation exchange (12, 29). However, strong cation exchange media are not suitable for quaternary amines, because elution by neutralization is difficult. Such elution can be achieved when weak cation exchange material is used and the acidic mobile phase is applied to the cartridge in the chromatographic method. Oasis weak cation exchange cartridges (Waters) contain mixed-mode weak carbonyl cation-exchange material, which retains strong bases such as MNs, at pH >5, permitting the cartridge to be washed thoroughly with both water and 100% acetonitrile without elution of the analytes of interest.

The use of HILIC for the analysis of polar bases provides enhanced analytical sensitivity compared with

Table 2. Mean recoveries of the XLC-MS/MS method for plasma free MN, NMN, and 3-MT.

Compound	Recovery analyte, % (range)	Recovery IS, % (range)
MN		
Low	98.9 (97.3–99.6)	99.0 (98.3–99.4)
Medium	97.2 (94.4–99.5)	97.2 (95.9–98.8)
High	97.3 (95.8–98.6)	97.3 (96.1–98.6)
NMN		
Low	94.6 (90.7–99.1)	96.1 (93.7–98.3)
Medium	83.5 (76.7–95.2)	83.1 (77.8–94.6)
High	81.6 (74.5–93.7)	82.0 (75.0–89.8)
3-MT		
Low	91.1 (85.2–96.0)	96.6 (91.3–99.0)
Medium	90.4 (81.4–96.6)	94.6 (92.2–98.4)
High	96.6 (92.8–98.5)	96.7 (89.9–98.0)

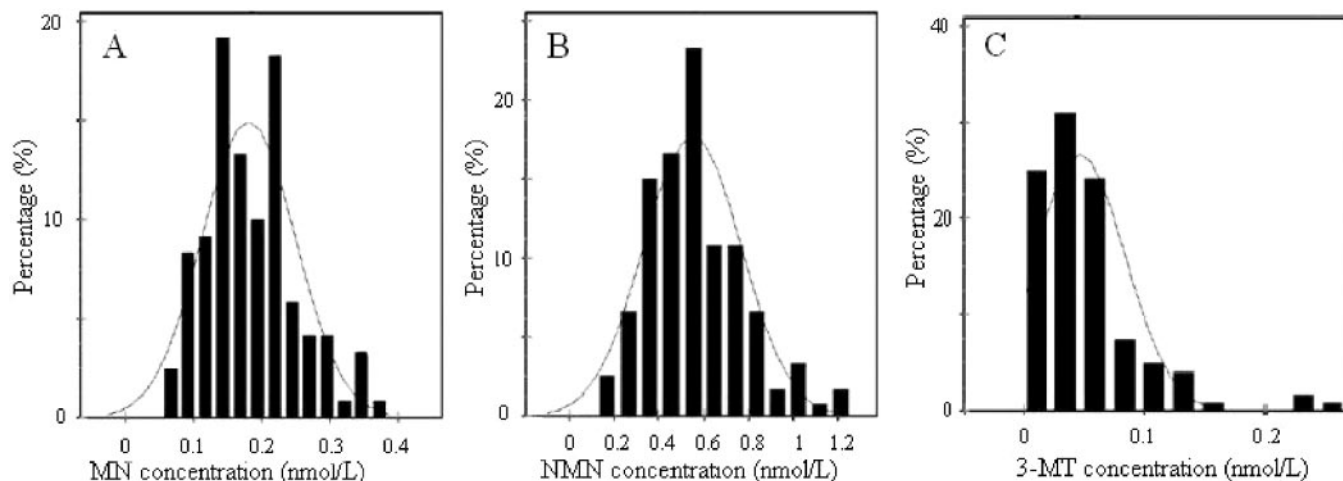


Fig. 3. Distribution of fractionated plasma free MNs in reference samples from 120 healthy individuals in nmol/L.

(A), MN; (B), NMN; (C), 3-MT.

traditional reversed-phase methods when using electrospray ionization. For the desolvation process, an organic solvent is more efficient, and with HILIC, MNs are eluted in a high proportion of organic solvent (>80%). The principal is normal-phase separation in a reversed-phase manner with a polar stationary phase and an aqueous-organic mobile phase. This mode of chromatography is especially suitable for the separation of polar compounds from possible matrix interferences (31). With MS/MS detection, high sensitivity is achieved, because unique parent-daughter ions are used for qualification and quantification. Interference with the same MRM transitions is eliminated chromatographically, whereas additional qualifiers enhance specificity of the detection method.

Correlation parameters show similarity between HPLC-ECD and XLC-MS/MS. However, XLC-MS/MS results in slightly higher concentrations than obtained with HPLC-ECD, which might be explained by the use of different calibration methods and internal standards. Ref-

erence intervals with XLC-MS/MS for MN and NMN (0.07–0.33 and 0.23–1.07 nmol/L, respectively) are in accordance with the ranges (0.05–0.47 and 0.12–1.1 nmol/L) measured by offline LC-MS/MS (16). These limits are slightly lower for MN and higher for NMN than determined by the HPLC-ECD method (0.06–0.63 and 0.12–0.73 nmol/L) (13). The difference in the upper reference limits for plasma free MN and NMN may be attributable to difference in internal standards (HMBA for HPLC-ECD; deuterated MNs for XLC-MS/MS) or sample collection position (sitting or supine).

The XLC-MS/MS method shows excellent linearity, and recoveries are consistent. The MNs are stable in plasma at 4 and 10 °C up to 7 days, which is in accordance with previous findings (27). Extended storage is possible at –20 °C, because at least 3 freeze-thaw cycles had no influence on plasma MN concentrations.

The method allows reproducible quantification of plasma MNs. Analytical variation is <10% (except for low 3-MT concentrations), which is lower than in offline methods, owing to automation of the sample preparation (16, 32). Biological variation exceeds analytical variation for all 3 MNs.

Limits of quantification are decreased in comparison with non-MS methods (13) and detectable down to 0.05 nmol/L. In addition, the required plasma volume can be scaled down to 50 μ L, which enables measurement of samples from infants and neonates. 3-MT concentrations can still be below the quantification limit in healthy individuals. In contrast, a few controls show increased concentrations of 3-MT (data not shown), which implies the necessity of further research before 3-MT concentrations can be used for the diagnosis of dopamine-dependent diseases such as extraadrenal paraganglioma (33).

Plasma samples from 10 histologically proven pheochromocytoma patients all showed NMN concentrations

Table 3. Plasma free metanephrine MN concentrations (nmol/L) in 10 patients with histologically proven pheochromocytoma.^a

Patient	MN	NMN	3-MT
1	17.24^b	18.03	0.76
2	17.93	11.92	0.10
3	2.04	6.78	0.05
4	0.14	4.05	0.03
5	0.09	56.80	2.63
6	0.11	70.10	2.62
7	14.62	5.41	64.89
8	1.70	12.59	0.07
9	3.58	13.24	0.15
10	15.18	59.44	0.47

^a Plasma concentrations are given in nmol/L. Reference limits are 0.07–0.33 nmol/L for MN, 0.23–1.07 nmol/L for NMN, and <0.17 nmol/L for 3-MT.

^b Boldface values: value exceeds upper reference limit.

that considerably exceeded reference limits. MN and 3-MT concentrations were increased in some patients.

This automated quantification of plasma free MNs has been used successfully for 6 months in the routine biochemical analysis in our laboratory, in addition to the urinary analysis of MNs for the diagnosis of pheochromocytoma. XLC-MS/MS is a promising method that enables automated, high-throughput, accurate quantification of several other clinical important biomarkers.

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