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A high sensitivity LC-MS/MS method for measurement of 3-methoxytyramine in plasma and associations between 3-methoxytyramine, metanephrines, and dopamine

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ABSTRACT

Introduction: Diagnosis of pheochromocytoma and paraganglioma (PPGL) is aided by the measurement of metanephrine (MN) and normetanephrine (NMN). Research suggests that 3-methoxytyramine (3MT), a dopamine (DA) metabolite, may serve as a biomarker of metastasis in patients with paraganglioma. Considering the very low endogenous plasma 3MT concentrations (<0.1 nM), highly sensitive and specific methods for 3MT are needed.
Methods: We developed a simple method for measurement of 3MT. Sample preparation was performed using solid phase micro-extraction with the eluates injected directly onto the LC-MS/MS. Data acquisition was performed in multiple reaction monitoring mode with an instrumental analysis time of 3 min per sample. We evaluated the method's performance and analyzed samples from healthy individuals and pathological specimens.
Results: The limit of quantitation and upper limit of linearity were 0.03 nM and 20 nM, respectively. The intra-/inter-day imprecision for pooled plasma samples at concentrations of 0.04 nM, 0.2 nM, and 2 nM was 10.7%/18.3%, 4.5%/8.9%, and 3.1%/0.9%, respectively. Among samples with MN, NMN, or both MN and NMN above the reference intervals (RIs), 0%, 16% and 46%, respectively, showed 3MT greater than the proposed upper RI value of 0.1 nM; 12% of samples with DA above the RI had 3MT above 0.1 nM.
Conclusions: The developed method allowed accurate quantitation of 3MT in patient samples and would provide valuable information to clinicians diagnosing or monitoring patients with PPGL. High 3MT concentrations in patient samples with MN and NMN within the respective RIs may alert clinicians of the possibility of a DA-producing tumor.

1. Introduction

Pheochromocytomas and paragangliomas (PPGLs) are two types of neuroendocrine tumors that produce catecholamines from intra-adrenal or extra-adrenal chromaffin cells, respectively [1]. Metanephrine (MN) and normetanephrine (NMN), known collectively as metanephrines, are metabolites of the catecholamines, epinephrine and norepinephrine, respectively, produced by catechol-*O*-methyltransferase (COMT) catalyzed *O*-methylation [2]. Measurement of these metabolites using liquid-chromatography with mass spectrometry or electrochemical detection is recommended for the detection and diagnosis of PPGLs [3,4]. However, use of MN and NMN does not allow for the detection of dopamine (DA)-producing forms of these tumors. Although dopamine-producing tumors are very rare (~135 cases have

Abbreviations: 3MT, 3-methoxytyramine; 3MT-d_4, deuterated 3-methoxytyramine; CE, collision energy; CI, confidence interval; COMT, catechol-*O*-methyltransferase; CV, coefficient of variation; CXP, collision cell exit potential; DA, dopamine; DBH, dopamine-β-hydroxylase; DP, declustering potential; EDTA, ethylenediaminetetraacetic acid; HCl, hydrochloride; HPLC, high-performance liquid chromatography; IQR, interquartile range; IS, internal standard; LC-MS/MS, liquid-chromatography tandem mass spectrometry; LOQ, limit of quantification; MAO, monoamine oxidase; MN, metanephrine; MN-d_3, deuterated metanephrine; NMN, normetanephrine; NMN-d_3, deuterated normetanephrine; PPGL, pheochromocytoma and paraganglioma; RI, reference interval; SD, standard deviation; *SDHx*, succinate dehydrogenase genes; SPE, solid phase extraction.

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been reported between 1980 and 2012), they may be underdiagnosed or misdiagnosed due to the lack of availability of biochemical tests enabling their detection [5,6]. For the detection of DA-producing tumors, research shows that measurement of the primary metabolite of DA, 3-methoxytyramine (3MT, Supplemental Fig. 1), may be diagnostically useful [6–8]. The proposed upper limit of the reference interval (RI) for 3MT from the literature is 0.1 nM [7–9], thus methods for measurement of 3MT should be highly sensitive.

Availability of 3MT concentrations, in addition to metanephrines, could be beneficial for a variety of patient populations. Concentrations of catecholamine metabolites, 3MT, MN and NMN, above the corresponding RI increase the post-test probability for detecting PPGLs and improve clinical sensitivity for detecting patients with head and neck paragangliomas [8]. In some patients, increased 3MT could be the only elevated catecholamine metabolite, as in a report of a patient undergoing screening due to a familial history of pheochromocytoma that resulted in detection of a rare cardiac paraganglioma [6,10]. Seventy percent of patients with a DA-producing tumor have mutations in succinate dehydrogenase genes (*SDHx*) and these mutations are often associated with tumors located at the base of the skull and in the neck [11,12]. Since *SDHx* mutations may be familial, monitoring patients with these mutations can ensure timely diagnoses of individuals with familial PPGLs. Additionally, patients with DA-producing tumors do not require preoperative α- or β-blockers usually given to control hypertension in patients with a more classic norepinephrine/epinephrine-producing pheochromocytoma [5]. Further, 3MT is often elevated in patients with metastatic PPGLs, thus 3MT could potentially be used as a biomarker of complications in patients diagnosed with PPGLs [13]. Finally, recent studies in children have shown that 3MT had 95–100% specificity for detecting neuroblastoma [14,15]. For all these reasons, clinical laboratories should consider offering 3MT and metanephrine analysis and part of this consideration will include selecting the specimen type. Measurement of free MN, NMN, and 3MT in plasma has been shown to have better diagnostic performance over measurement in urine in most patients and, therefore, plasma may be the preferred specimen type [16]. Blood samples also provide a greater convenience to patients over collection of a 24 h urine sample.

Since there is considerable potential clinical benefit to measuring 3MT, in addition to the metanephrines, our objective was to incorporate 3MT measurement into a previously developed liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for MN and NMN [17]. Additionally, we used the new method to measure the 3MT concentration in residual samples from patients with MN, NMN, both MN and NMN, or DA elevated above the corresponding RIs, as well as in samples from healthy individuals.

2. Materials and methods

2.1. Standards and chemicals

The following materials were used to prepare calibration standards, internal standards (IS), and quality control samples: 3MT HCl in methanol and 3MT-d_4 HCl in methanol (Cerilliant, Round Rock, TX); DL-MN HCl powder (International Laboratory USA, South San Francisco, CA); DL-NMN HCl powder (Sigma Aldrich, St. Louis, MO); DL-MN-d_3 HCl powder and DL-NMN-d_3 HCl powder (Medical Isotopes, Inc., Pelham, NH). Deionized water was prepared using a Barnstead NANO-pure water system (Thermo Fisher Scientific, Inc., Waltham, MA). Chemicals and solvents used include: ≥99.995% pure ammonium formate from Sigma Aldrich (St. Louis, MO), ≥99.7% pure glacial acetic acid and ≥99.9% pure methanol from J. T. Baker Chemical Co. (Phillipsburg, PA), and ≥99.9% pure acetonitrile from Honeywell, Burdick & Jackson (Muskegon, MI).

2.2. Calibrators, internal standards, and quality control samples

Calibrators with 3MT concentrations of 0.03, 0.1, 0.2, 1.0, and 5.0 nM were prepared by spiking SeraSub synthetic serum (CST Technologies, Great Neck, NY) with 3MT standard. SeraSub was assayed for the 3MT concentration, which was below the limit of quantification (LOQ). Stock IS solutions were prepared in 0.1 M acetic acid at concentrations of 5 μM of the deuterated compounds 3MT-d_4, MN-d_3, and NMN-d_3. The working IS were prepared in water at concentrations of 50 nM 3MT-d_4, 5 nM MN-d_3, and 5 nM NMN-d_3. Plasma controls containing MN and NMN within the RI were purchased from ChromSystems GmbH (Munich, Germany). Controls containing pathological concentrations of MN and NMN were prepared by spiking pooled, dialyzed plasma with MN and NMN. Residual proficiency testing material (The Royal College of Pathologists of Australasia Quality Assurance Programs, St. Leonards, NSW, Australia), stored for two years at −70 °C, was used as quality control for 3MT.

2.3. LC-MS/MS conditions

The LC-MS/MS system consisted of an Agilent series 1260 pump and heated column compartment (Agilent, Santa Clara, CA), and HTC PAL autosampler (LEAP Technologies, Morrisville, NC) connected to an AB Sciex 5500 tandem mass spectrometer (Framingham, MA). An Atlantis HILIC silica column (2.1 × 50 mm, 3.0 μm, Waters, Milford, MA) with an in-line filter assembly and frit (0.2 μm 2.1 mm, Waters, Milford, MA) maintained at 25 °C was used for the high-performance liquid chromatography (HPLC) separation. Mobile phase A consisted of 100% acetonitrile; mobile phase B was 100 nM ammonium formate in water (pH 3.0). The injection volume was 30 μL and the flow rate was 500 μL/min. The following gradient was used: 0–1.0 min. 5% to 20% B, 1.0–2.0 min. 20% B, 2.0–2.1 min. 20% to 5% B, 2.1–3.0 min. 5% B. The instrument was operated in multiple reaction monitoring, positive ion mode with a 1250 V Turbo Spray voltage, 700 °C ion source temperature, 10 V entrance potential, 30 psi curtain gas, 50 psi nebulizer gas (gas 1), 60 psi heated gas (gas 2), and collision gas setting of 8 psi. The dwell time for the mass transitions was 25 ms. The retention times, mass transitions, declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP) data for the analytes are summarized in Table 1.

2.4. Method validation

The LOQ was determined by analyzing a progressively diluted (with SeraSub) pooled plasma sample in triplicate. Linearity was evaluated by analyzing pooled plasma spiked with 3MT to concentrations of 20, 10, 5, 2.5, and 1.25 nM and measuring the samples in triplicate. Assay intra- and inter-day imprecision was determined by analysis of three pooled plasma samples in five replicates on three different days. The lowest evaluated pooled plasma samples contained endogenous 3MT (0.04 nM) and the other two pools were spiked with 3MT standard to concentrations of 0.2 and 2.0 nM. Accuracy was determined by measuring residual outdated proficiency testing samples containing MN, NMN, and 3MT stored frozen at −70 °C. The acceptability criteria for the method's performance were accuracy within ±15% and imprecision for the LOQ of ±20% coefficient of variation (CV). The acceptability criterion for the ratio of mass transitions was set to ±30% [18]. Matrix effects were assessed by comparison of 3MT peak area observed in analysis of 3MT-free patient plasma samples and SeraSub spiked to the same 3MT concentration.

2.5. Samples

A method evaluation was performed using de-identified residual aliquots of plasma specimens (EDTA, sodium or lithium heparin) submitted for routine testing to ARUP Laboratories (Salt Lake City, UT). Available sample-related information included the age, sex, and concentrations of

Table 1
Mass spectrometry method mass transitions and instrument settings.

Compound	Retention Time (min) median (IQR)	Precursor *m/z*	Product *m/z*	DP (V)	CE (V)	CXP (V)
3MT						
Quantifying peak	1.72 (1.71–1.75)	168.1	119.1	65	26	19
Qualifying peak		168.1	91.1	65	33	19
3MT-d_4						
Quantifying peak		172.1	123.1	65	26	19
Qualifying peak		172.1	95.1	65	33	19
MN						
Quantifying peak	1.77 (1.76–1.79)	180.1	148.1	80	24	17
Qualifying peak 1		180.1	165.1	80	23	27
Qualifying peak 2		180.1	120.1	80	24	17
MN-d_3						
Quantifying peak		183.1	151.1	80	24	21
Qualifying peak 1		183.1	168.1	80	33	25
Qualifying peak 2		183.1	123.0	80	20	17
NMN						
Quantifying peak	1.78 (1.77–1.81)	166.1	134.1	80	22	15
Qualifying peak		166.1	106.1	80	23	18
NMN-d_3						
Quantifying peak		169.1	137.1	80	14	15
Qualifying peak		169.1	109.1	80	17	15

DP, declustering potential; CE, collision energy; CXP, collision cell exit potential; IQR, interquartile range.

metanephrines or DA. Samples from self-reported healthy adult volunteers were used for evaluation of the distribution of 3MT concentrations. The specimens were collected in sodium heparin tubes, centrifuged promptly, plasma was separated and stored at −20 °C in light-protected transport tubes until the day of testing. All specimens were de-identified and handled according to guidelines approved by the Institutional Review Board of the University of Utah (IRB #7275, #7740).

2.6. Sample preparation

A blank (water), calibrators, quality control, and patient samples were prepared in the same manner. The approach for sample preparation and instrumental analysis was similar to the method developed by Petteys et al. with modifications made to enhance the analytical sensitivity for 3MT [17]. ISs were added to 400 μL of sample in a microcentrifuge tube giving a final volume of 730 μL and final concentration of 2 nM for each IS. The samples were vortexed for 30 s and centrifuged at 2500 g for 10 min. The supernatant (650 μL) was quantitatively transferred to a 96-well Oasis WCX μElution plate (2 mg sorbent per well, 30 μm, Waters, Milford, MA) for solid phase extraction (SPE). The SPE adsorbent was preconditioned with methanol and water. The extraction was performed on a positive pressure manifold (Biotage Pressure+, Biotage, Charlotte, NC); a flow rate of approximately one drop per second was used to load the samples, wash the adsorbent, and elute the extracts. After the samples were loaded, the adsorbent was washed sequentially with 500 μL each of water, methanol, and 0.1% formic acid in acetonitrile. Samples were eluted from the adsorbent into a collection plate (part AHO-7194, Phenomenex, Torrance, CA) using two applications of 50 μL of 2% formic acid in acetonitrile. The plate was sealed (part AHO-8598, Phenomenex, Torrance, CA) and the samples were analyzed by LC-MS/MS.

2.7. Data analysis

Data acquisition and processing were performed using Analyst software, version 1.6.2 and MultiQuant, version 3.0 (AB Sciex, Framingham, MA). EP Evaluator, version 10.3.0.556 (Data Innovations, LLC, South Burlington, VT) was used to calculate the LOQ and assess linearity. Intra- and inter-day imprecision was calculated using Excel 2016 v. 16.16.2 for Mac (Microsoft, Redmond, WA) according to the equations provided in the Tietz Textbook of Clinical Chemistry and Molecular Diagnostics [19]. Prism version 8 for Mac (GraphPad Software, 2010, LaJolla, CA) was used for patient data analysis and graph preparation. The comparison of 3MT concentrations among groups was performed using the Kruskal-Wallis and Dunn's tests. For analysis, samples containing concentrations below the LOQ of the assay were assigned a value of one-half of the LOQ and concentrations above the upper limit of the analytical measurement range were assigned the value of the upper limit of linearity. Mean age was compared using one-way analysis of variance with Tukey's multiple comparisons. RIs for MN, NMN and DA in plasma were <0.50, <0.90, and <0.14 nM, respectively [17].

3. Results

3.1. Method validation

Example chromatograms of 3MT extracted from a calibrator and a patient sample are shown in Fig. 1 and the method performance evaluation results are summarized in Table 2. Although the proficiency samples had been stored frozen for approximately two years, MN, NMN, and 3MT results were within the expected range supporting their likely stability and the accuracy of this method.

No carryover was observed after injection of samples containing 5.0 nM of 3MT. Prior to analysis, all samples, calibrators, controls, and the RCPA samples were stored at −20 °C or below. The acceptable results for the RCPA samples suggest that 3MT is stable for up to 2 years when stored at −70 °C and can undergo at least 3 freeze–thaw cycles. The calibration standards were stable for at least two months. At three evaluated concentrations [0.2 nM (3 days, 5 replicates per day); 1.0 nM (1 day, 2 replicates); 5.0 nM (1 day, 2 replicates)], the 3MT peak area in the extracts of plasma samples was on average 49.7% lower than in SeraSub (0.2 nM: −44.1%, 1.0 nM: −47.9%, 5.0 nM: −57.0%). Considering that chromatographic peaks of 3MT and 3MT IS fully coelute (Fig. 1), the observed matrix effects would have no effect on the quantitative performance of this method.

3.2. Patient and control group demographics and sample 3MT concentrations

Using this method, we measured 3MT concentrations in patient samples with elevated MN (n = 17), NMN (n = 25), both MN and NMN (n = 26), and DA (n = 24), as well as in samples with concentrations of MN and NMN within the RI (n = 27), and samples from healthy donors (n = 10). The distribution of 3MT concentrations for these groups is shown in Fig. 2. Since the 3MT results for the healthy adults and MN and NMN within the RI were below 0.1 nM, this verifies the previously reported RIs and supports adequate performance of the assay in our patient population [7–9]. The age distribution in the healthy adult group (mean age 33 y) differed significantly from the other groups included in this study (mean age 61 y); there was no significant difference in age among the other groups.

Forty-six percent of samples in the group with elevated MN and NMN (concentrations above the RI) had 3MT concentrations greater than 0.1 nM, as compared to 16% in the group with elevated NMN, 12% in the group with elevated DA, 4% in the group with MN and NMN within the

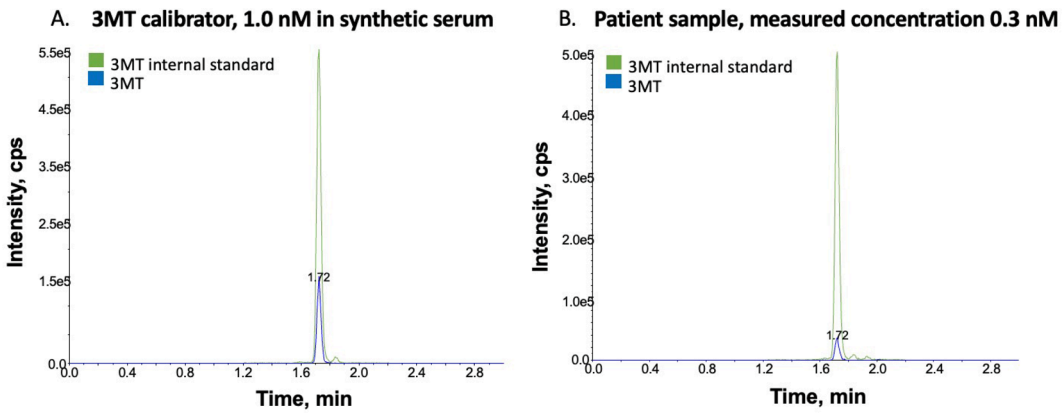


Fig. 1. Representative 3MT chromatograms. A. Calibration standard containing 1.0 nM 3MT. B. Patient sample with a measured concentration of 0.3 nM 3MT, MN concentration 0.28 nM (within the RI), and NMN concentration 3.54 nM (above the RI).

Table 2
Summary of the method performance evaluation results for 3MT.

Performance characteristic	Result
Limit of quantitation (LOQ) (nM)	0.03
CV at LOQ (95% CI)	11.7 (6.3–15.9)
Linear range (nM)	0.03–20.0
Recovery, average ± SD (range)* (%)	100.4 ± 4.7
Calibration curve data	(84.1–107.8)
Average slope ± SD	0.496 ± 0.064
Average y-intercept ± SD	0.004 ± 0.005
Average R^2 ± SD	0.999 ± 0.0002
Imprecision CV (%): Intra-day / Inter-day	
0.04 nM	10.7 / 18.3
0.22 nM	4.5 / 8.9
2.10 nM	3.1 / 0.9
Accuracy, median SDI (range), (n = 4 samples, 9 measurements)#	0.99 (0.36–1.25)

nM = nanomolar; CI = confidence interval; CV = coefficient of variation; LOQ = limit of quantification; SD = standard deviation; SDI = standard deviation index.
* Determined from data on performance of calibration standards: 0.03, 0.1, 0.2, 1.0, 5.0 nM (n = 6 calibration curves).
# 3MT concentrations: 0.35, 0.94, 0.97, and 1.16 nM.

RI (one patient sample in this group had a 3MT concentration of 4.3 nM, Fig. 2), and none in the group with elevated MN and the healthy donor samples. In a sub-analysis of the patient samples with elevated concentrations of DA, the patterns of elevated 3MT with elevated MN and/or NMN were different, as compared to the other groups. Only one sample with elevated DA and 3MT had elevated NMN. In the group with elevated DA, there were 10 patient samples with elevated NMN and 2 with elevated MN and NMN, but none of these samples had elevated 3MT. This likely reflects the different patient populations for which each test, metanephrines versus dopamine, was ordered and the decreased pre-test probability of PPGL for patients with orders for dopamine. This observation and hypothesis must be confirmed in future studies.

3.3. Associations of 3MT concentrations with metanephrines, DA, and age

Fig. 3 demonstrates association of 3MT concentrations with concentrations of MN, NMN and DA in the study samples. The strongest association was observed between concentrations of 3MT and NMN (Spearman rho (ρ) = 0.57, p < 0.0001, n = 129) followed by DA (ρ = 0.42, p = 0.04, n = 24) and MN (ρ = 0.30, p = 0.0005, n = 129). A significant association was also observed between MN and NMN (ρ = 0.50, p < 0.0001). Neither MN nor NMN was associated with DA concentration. No associations were observed between concentrations of 3MT, MN, or DA with age or sex. The distributions of NMN

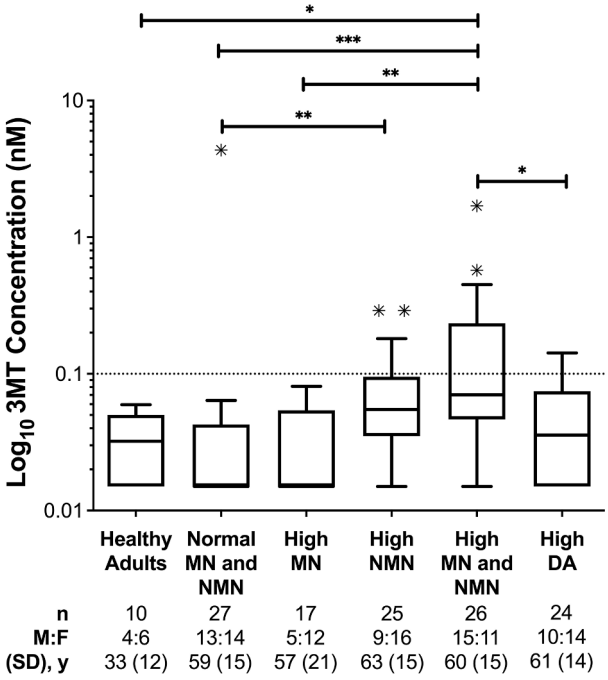


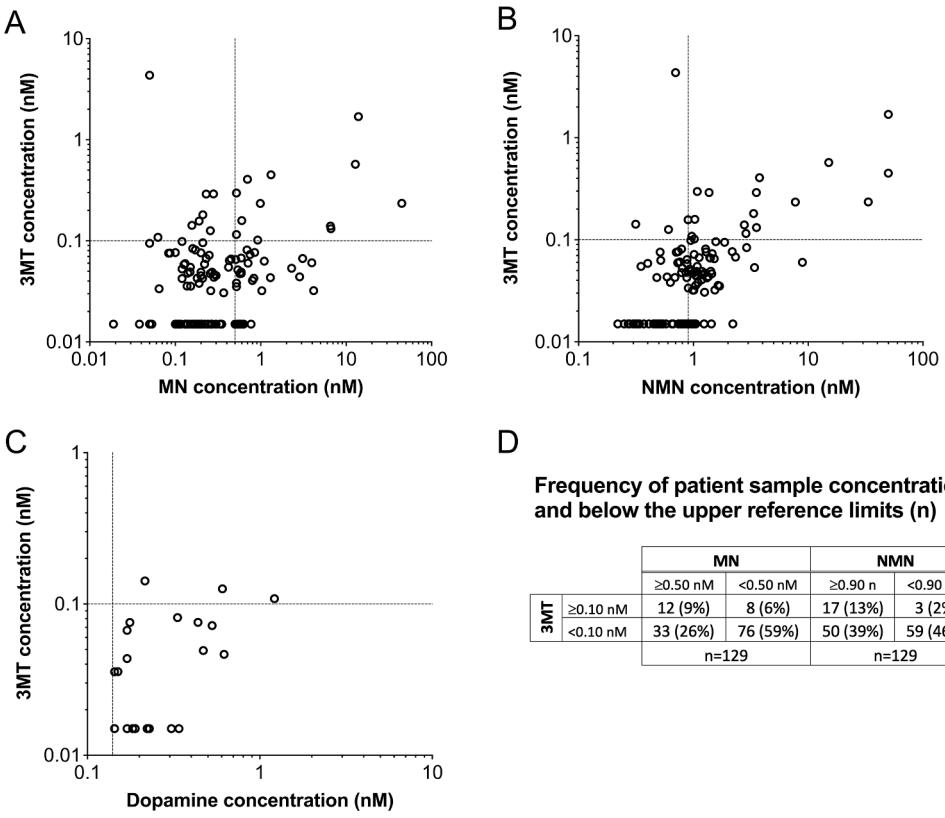
Fig. 2. Distribution of 3MT concentrations among groups including samples from healthy adult volunteers and patients with MN and NMN within the RI (normal), MN and/or NMN and DA above the RI (high). The demographics shown below each group are sample size (n), male to female ratio (M:F) and mean age (SD) in years. Data are presented as Tukey boxplots with stars indicating statistical outliers. The 0.1 nM proposed upper limit of the RI for 3MT is shown as a dotted line. 3MT concentration was compared among groups using the Kruskal-Wallis test with Dunn's test to calculate adjusted p-values for multiple comparisons. Mean age was compared using one-way analysis of variance; adjusted p-values were determined using Tukey's multiple comparisons test. *p < 0.05, **p < 0.01, ****p < 0.0001.

concentrations were not different between men and women, while they were positively associated with age (ρ = 0.19, p = 0.006; data not shown).

4. Discussion

4.1. Method performance

We developed an LC-MS/MS method for measurement of 3MT; the method is similar to a method for measurement of MN and NMN



		MN		NMN		DA
		≥0.50 nM	<0.50 nM	≥0.90 n	<0.90 nM	>0.14 nM
3MT	≥0.10 nM	12 (9%)	8 (6%)	17 (13%)	3 (2%)	3 (12)
	<0.10 nM	33 (26%)	76 (59%)	50 (39%)	59 (46%)	21 (88)
		n=129		n=129		n=24

Fig. 3. Associations between concentrations of 3MT vs. MN (A, n = 129); 3MT vs. NMN (B, n = 129); 3MT vs. DA (C, n = 24). The number of patient samples with observed concentrations above or below the corresponding upper RI are summarized in panel D. Graph x- and y-axes are plotted using a $\log_{10}$ scale. The dotted lines indicate the upper reference limits for each analyte.

developed and used by our laboratory [17]. The instrumental analysis was modified to allow for detection of 3MT and the sample preparation was optimized for enhancement of sensitivity and specificity for 3MT measurement. Changes in the sample preparation, as compared to Petteys et al., included use of a larger volume of sample and more extensive SPE adsorbent washes prior to elution [17]. The modifications enhanced analytical sensitivity to achieve an LOQ of 0.03 nM with a CV under 20%; thus, the assay is sufficiently sensitive for quantitation of endogenous concentrations of 3MT characteristic of healthy individuals. Several MS-based methods for 3MT have been reported, which differ in the required sample volume and approaches used for sample preparation, chromatographic separation, MS detection, run time, sensitivity and analytical measurement range [20–25]. As compared to other published methods, advantages of this method include simple sample preparation consisting of a single-step SPE with direct injection of the SPE eluate for the instrumental analysis, high sensitivity and specificity, fast instrument analysis time, and clinically relevant analytical measurement range. Although this assay is sufficiently sensitive for detecting low 3MT concentrations, the required sample volume could be too high to support measurement of 3MT in samples from infants or children suspected of PPGL or neuroblastoma. Use of online SPE methods could allow analysis of smaller sample volumes [26]. Further, enhancement of analytical sensitivity could be achieved through use of a derivatization step [24,26], but this is not always necessary for detecting endogenous 3MT concentrations in healthy patients as shown by this method and others published recently [23,25].

Given the Endocrine Society's recommendation for lifelong monitoring of patients treated for PPGLs to detect metastases or recurrent disease [4], the availability of an analytically sensitive and fast method for 3MT analysis will be beneficial for patient care. Currently, plasma determination of 3MT does not appear to be available in most laboratories in the US. Our data demonstrate that 3MT can be incorporated as an additional analyte in assays for plasma MN and NMN.

In addition to the high sensitivity, our method is not subject to interference, which was reported in some earlier methods. Twentyman et al. have reported that crosstalk may occur between 3MT and MN, and to a lesser extent NMN, due to the production of an *m/z* 151 fragment in the mass spectrometer ion source from MN or NMN that mimics 3MT, which could result in falsely elevated concentrations of 3MT (up to 2% of the MN concentration) [27]. Crosstalk occurs when in-source fragmentation of one or more structurally related analytes produces product ions in common with the analyte of interest, while the chromatographic peaks of the analytes are not resolved. This method avoids two significant factors potentially leading to crosstalk. First, the common *m/z* 151 product ion is not used as a precursor ion for 3MT determination [20–22,25,28]. Therefore, production of any *m/z* 151 product ion in the source from MN will not contribute to the 3MT concentration. Second, and likely most importantly since a few different product ions are common amongst the metanephrines and 3MT, the 3MT peak is chromatographically resolved from the peaks of MN and NMN in this assay [27]. For these reasons, MN is not expected to interfere with measurements of 3MT in the current method. Absence of interference from MN is supported by the observation that the upper quartile of the distribution of 3MT concentrations (Fig. 2) is below the proposed upper reference limit of 0.1 nM in samples from the healthy donor group, in the majority of samples from individuals with MN and NMN within the respective RIs, and in the samples with elevated MN.

Peitzsch et al. reported potential for interference of 3-*O*-methyldopa with 3MT in patients treated with L-dopa and in patients with renal disease [29]. The interference is likely a result of the in-source decarboxylation of the 3-*O*-methyldopa molecule, which results in formation of 3MT [29]. Such interference could happen in methods in which 3-*O*-

methyldopa is coextracted from samples along with 3MT and the two peaks are not chromatographically resolved. In a few of the analyzed samples in this study an unknown peak co-eluted with the 3MT peak and prevented accurate 3MT quantitation (8 samples with 3MT $\geq$ 0.1 nM and 6 samples with 3MT $<$ 0.1 nM). The presence of the interference was detected through evaluation of the ratios of the mass transitions. Additional studies are required to identify the interfering substance.

4.2. Distribution of 3MT concentrations in healthy adults

In samples from healthy adults and in patient samples with MN and NMN concentrations within the RI, 3MT concentrations were below the proposed upper reference limit of 0.1 nM [7–9]. As discussed by Peitzsch et al., some of the earlier studies have reported an upper reference limit for 3MT of 0.17–0.18 nM suggesting an overestimated concentration [28]. Possible explanations for the higher reported upper RI limits in the earlier studies could be MN interference with 3MT, which was unrecognized, or alternatively dietary contribution of 3MT [27].

4.3. Association of 3MT concentration with age and concentrations of metanephrines and DA.

Evaluation of the associations between concentrations of 3MT and age, sex, and concentrations of metanephrines and DA was performed using patient plasma samples containing concentrations of MN, NMN, both MN and NMN, or DA above the corresponding RI. In agreement with the earlier reports, NMN concentrations were positively associated with age (data not shown) [8,28,30,31]. Our data did not show correlation of 3MT with age, whereas a weak association has been reported in larger cohorts [8,9,28]. In a recent study by Eisenhofer et al., age specific RIs for 3MT were not recommended [9]. Unlike in adults, 3MT does vary with age in infants and children up to 3 years of age [32]. Thus, clinical laboratories offering 3MT testing in samples from infants or children should provide age specific pediatric RIs.

Assessment of the association between concentrations of 3MT and metanephrines or DA demonstrated the strongest association with NMN, followed by DA and MN. Elevated 3MT concentrations were most often observed in patient samples with elevated MN and NMN followed by those with elevated NMN only and lastly with elevated DA (Fig. 3). The presence of elevated MN, NMN, and 3MT is consistent with PPGLs and higher increases in concentrations may be seen in low-risk patients presenting with symptoms as compared to high-risk patients with incidentalomas, previous disease history, underlying mutations, possibly due to earlier detection in the high-risk patients through active screening [16]. Association of 3MT concentrations with concentrations of MN, NMN, and DA have been assessed in few publications. Rao et al. reported association between concentrations of 3MT and NMN, which is comparable to the data shown in Fig. 3B [8]. Overall, studies indicate that elevated 3MT has improved the diagnostic sensitivity for head and neck PPGLs when considered in addition to MN and NMN and has been associated with metastatic disease and poor prognosis in children with neuroblastoma [8,13,33], but the association of the relative changes in the metanephrines and 3MT with respect to each other and disease outcomes has infrequently been explored [13]. While the current study is not able to interrogate these interrelationships with respect to patient outcomes, doing so in future studies might be revealing.

Considering that 3MT is a direct metabolite of DA (Supplemental Fig. 1), it is noteworthy that we observed that concentrations of 3MT were associated more strongly with NMN, a downstream metabolite of DA (Fig. 3). In agreement with our results, Rao et al. reported that in patients with PPGLs and elevated NMN or both MN and NMN, concentrations of 3MT were elevated also [8]. One possible explanation for the association between 3MT and NMN concentrations may be related to the production of the metabolites locally in the tumor [34]. Chromaffin cells favor production and storage of epinephrine and norepinephrine rather than dopamine. Subsequently, catecholamine metabolism to metanephrines occurs predominantly intracellularly, rather than from circulating catecholamines, via the membrane-bound form of COMT, which is found in abundance in the chromaffin cells of the adrenal medulla or pheochromocytoma tumor [35]. The preferential production of the metanephrines over 3MT may explain the stronger association of 3MT with NMN (or both MN and NMN) in that 3MT may be produced only after a sufficient excess of dopamine is present causing increased activation of the 3MT production pathway. Although dopamine-β-hydroxylase enzyme (DBH) activity is increased in pheochromocytomas [36], a correlation between DBH activity and concentrations of DA and norepinephrine was not found in a previous study [37]. Of note, in our study, in samples with elevated MN only (above the RI), 3MT was not elevated, which was also reported by Rao et al. [8].

Measurement of 3MT along with MN and NMN in plasma samples has been reported to improve the diagnostic sensitivity for PPGL and metastases, such that a correct diagnosis could be established in patients who might have otherwise been missed (with MN and NMN within the respective RIs but with elevated 3MT) [16]. This could apply to one patient sample analyzed in the current study, which had MN and NMN within the RI, but the 3MT concentration was extremely elevated. The elevated 3MT concentration may be attributable to the presence of a predominantly DA-producing tumor in that patient [6], though we did not have access to the clinical information to confirm the diagnosis. Alternatively, a deficiency or variant encoding polymorphism in one of the enzymes involved in DA metabolism, such as DBH or monoamine oxidase (MAO; regulates conversion of 3MT to homovanillic acid), may result in elevated 3MT [38–40]. The presence of DBH variants tends to result in concentrations of MN and NMN that are decreased, or normal NMN if treated, and MAO variants can result in MN and NMN being elevated [38–40]. Considering the above, as well as the fact that 3MT may guide decisions for genetic testing [11], measurement of 3MT in clinical samples (in addition to plasma free metanephrines) should be considered.

4.4. Limitations of the study

In this study, we utilized residual aliquots of de-identified patient samples that were submitted to a reference laboratory; therefore, access to patient charts to confirm the diagnoses and review clinical history was not possible. However, the study samples containing concentrations of metanephrines and DA above the corresponding RIs were likely from patients with pathological conditions, but a bias in sample selection may have occurred due to not using age specific NMN RIs [9].

Pre-analytical factors, including fasting status and patient position at the time of specimen collection, are known to influence the results of 3MT and metanephrines testing, respectively [3,4]. Because the samples in this study were sent to a reference laboratory for testing, specific information on patient preparation or specimen collection was not available. However, the 3MT concentrations with MN and NMN within the reference interval in this study were below 0.1 nM, similar to the healthy subjects. This suggests the collection instructions for position were most likely followed for patients with MN and NMN test requests and that 3MT $<$0.1 nM is typical for healthy patients. With respect to fasting status, a diet consisting of catecholamine-rich foods could significantly increase plasma 3MT concentrations in non-fasting specimens, while having less effect on plasma MN and NMN, although the study where this was reported involved meals that were excessively catecholamine-rich and, thus, may not be applicable to the general population [41]. Since many common foods such as bananas, nuts, fruit and fruit drinks contain catecholamines, there is a possibility of positive bias in the 3MT results observed in this study due to lack of fasting. However, it is expected that any impact on 3MT concentrations was likely negligible because the highest 3MT concentration was well below 0.1 nM for the healthy adults and those specimens with MN and NMN within the reference interval (except for one patient who's elevated 3MT was not likely due to diet alone given the degree of elevation (Fig. 2)).

5. Conclusions

We developed a sensitive and specific method for the quantitative measurement of 3MT in plasma samples that could be incorporated in an established LC-MS/MS assay for metanephrines, thus reducing potential barriers related to offering 3MT measurements in clinical laboratories. The assay performance was evaluated using samples from healthy donors and samples with elevated concentrations of metanephrines and DA. The 3MT concentrations observed in healthy subjects were lower than in some earlier reports. One possible explanation could be related to the possible MN interference with 3MT in the earlier studies, which was avoided in the current method through selection of more specific mass transitions and chromatographic separation to resolve the MN and 3MT peaks. Assessment of the association between concentrations of 3MT with metanephrines and DA demonstrated the strongest association of 3MT with concentrations of NMN. While 3MT was not increased in specimens with elevated MN, 3MT was elevated in approximately half of the patient samples with both MN and NMN elevated, 16% of patient samples with elevated NMN, and 12% of patients with elevated DA. Finally, 3MT concentrations above the RI may alert clinicians of the possibility of a DA-producing tumor when MN and NMN are within the RI, which may have significant impact on patient care and outcomes.

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CRediT authorship contribution statement

Laura Smy: Validation, Methodology, Formal analysis, Investigation, Writing – original draft, Visualization, Project administration, Funding acquisition. **Mark M. Kushnir:** Conceptualization, Methodology, Validation, Resources, Visualization, Supervision. **Elizabeth L. Frank:** Conceptualization, Resources, Supervision.

Conflicts of Interest

None of the authors has any conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmsacl.2021.08.001.

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