HHS Public Access

Author manuscript

J Chromatogr B Analyt Technol Biomed Life Sci. Author manuscript; available in PMC
2019 March 04.

Published in final edited form as:

J Chromatogr B Analyt Technol Biomed Life Sci. 2018 November 01; 1099: 83–91. doi:10.1016/j.jchromb.2018.09.012.

Comprehensive, robust, and sensitive UPLC-MS/MS analysis of free biogenic monoamines and their metabolites in urine

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Abstract

Biogenic monoamines, including catecholamines and serotonin are important hormones and neurotransmitters. Abnormal urinary levels of biogenic monoamines and their metabolites are associated with smoking, neuroendocrine tumors, as well as neurological and cardiovascular diseases. Measurements of free biogenic monoamines and their metabolites have been challenging because of low concentrations in complex biological matrices. Current methods require extensive enrichment and removal of interfering substances and can analyze only basic or acidic compounds in a single run. We developed a simple and robust dilute-and-shoot method capable of measuring 10 analytes, including free biogenic monoamines and their metabolites in human urine. The assay enables sensitive measurements of analytes within expected sample concentration ranges. To assess the assay's efficacy, we measured urinary levels of free biogenic monoamines and their metabolites in 255 non-smokers and 191 smokers. Our data show that while smokers had significantly higher urinary levels of free catecholamines and metanephrines, there was a decrease in levels of biogenic amine metabolites synthesized through the monoamine oxidase pathway - homovanillic acid and vanillylmandelic acid. The method could be used for high throughput measurement of the range of free biogenic amines and their metabolites in urine under a variety of different conditions.

Keywords

Catecholamines; Metanephrines; Serotonin; Dopamine; Tobacco; Cigarettes

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Declaration of interest

Authors do not have any conflict of interest.

1. Introduction

Biogenic monoamines such as catecholamines and serotonin are neurotransmitters and neuromodulators that regulate cardiovascular function as well as cognitive functions such as emotions, attention, addiction, appetite, and mood. Serotonin (5-HT) and catecholamines such as epinephrine (EPI) norepinephrine (NE) dopamine (DA) are also critical regulators of neurological function. Increased levels of catecholamines are associated with neuroendocrine tumors such as neuroblastoma [1–5] and pheochromocytoma [6–8], as well as a variety of other pathological conditions [9]. Abnormally high levels of catecholamines have also been linked with neurological disorders such as Parkinson's disease [10], schizophrenia [11], posttraumatic stress disorder [12]; hypertension [13–15], and myocardial infarction [16]. In addition, several studies have shown that the levels of these neurotransmitters are increased in the plasma and urine of individuals who use tobacco products [17–21].

The biogenic amines – EPI, NE, and DA are metabolized in the liver by the catechol-*O*-methyltransferase [22,23] to their corresponding methoxylated analogs – metanephrine (MN), normetanephrine (NMN), and 3-methoxytyramine (3-MT) (Scheme 1). Monoamine oxidase (MAO) then metabolizes MN, and NMN to 3-methoxy-4-hydroxyphenylglycolaldehyde (MOPEGAL), which is then further transformed to a more stable metabolite vanillylmandelic acid (VMA) via an aldehyde dehydrogenase catalyzed reaction [22,23]. MAO also catalyzes the biotransformation of 3-MT to 4-hydroxy-3-methoxyphenylacetaldehyde (HMA), which is then metabolized by aldehyde dehydrogenase to the stable metabolite homovanillic acid (HVA) [22,23]. In addition, MAO also catalyzes the conversion of 5-HT to 5-hydroxyindole-3-acetaldehyde (5-HIAL), which is then transformed to the stable product 5-hydroxyindole-3-acetic acid (5-HIAA) [22]. Hence, in addition to the measurement of parental biogenic amines, the measurement of their phase I metabolites provides complementary information regarding changes in biogenic amine metabolites. Stable metabolites of biogenic amines, such as VMA and HVA, for example are used as the biomarkers for the detection of neuroblastomas and similar tumors [4,5]. Similarly, 5-HIAA is used as a biochemical marker of several neuroendocrine tumors [9]. A large fraction of biogenic monoamines and metanephrines further undergoes phase II metabolism to yield respective sulfated and glucuronated forms, which are excreted in urine. [23]

Several bioanalytical approaches have been used to measure free biogenic monoamines in tissues as well as biological matrices such as serum, plasma, urine, and saliva [2,25–30]. A variety of analytical methods, including high-performance liquid chromatography (HPLC), or electrophoretic separation with UV [31] or fluorimetric [32] detection, have also been used to quantify free biogenic monoamines and their metabolites in the urine. However, hydrolysis and/or partial decomposition of analytes, and interferences from co-eluting species limit the specificity of these analyses. HPLC with electrochemical [18,33–37] or fluorescent detection (FLD) [26,38,39] improves the sensitivity of detection. The sensitivity and specificity of measuring free biogenic monoamines and their metabolites in the urine is enhanced by coupling HPLC or ultrahigh performance liquid chromatography (UPLC) with tandem mass spectrometry (MS/MS). Presence of the free amino group (Scheme 1) in

biogenic monoamines and metanephrines facilitates their protonation, and the protonated ions are analyzed by mass spectrometry in the positive ion mode. In contrast, metabolites of biogenic amines that possess a carboxyl moiety – prone to deprotonation – are typically measured in the negative ion mode. Therefore, the HPLC/UPLC-MS/MS assays are now often used to measure the basic biogenic monoamines [32,40,41], metanephrines [24,42], or their acidic downstream metabolites (VMA, HVA and 5-HIAA) [25,43]. However, current HPLC/UPLC-MS/MS assays for the characterization and the quantification of free biogenic monoamines and their metabolites have two major drawbacks: a) low abundance of free biogenic monoamines and their metabolites in the urine or other biofluids requires labor intensive sample cleanup and pre-concentration steps, such as solid phase extraction (SPE) [24,42,44,45], a step that is sometimes preceded by derivatization [46] or liquid-liquid extraction (LLE) [47]; and b) differences in chemical character of biogenic monoamines and their carboxylic acid metabolites restrain the choice of appropriate sorbent or solvent for the extraction of either the amines or carboxylates, but not both. Similar group specificity restrictions are present when chemical derivatization is used with dilute-and-shoot approaches [48], or in conjunction with SPE/LLE methods, to increase sensitivity [46]. This limits the analyses to the measurement of only 3–5 analytes in a single run. To overcome these limitations, we developed a novel, comprehensive, robust, and rapid UPLC-MS/MS assay of high sensitivity which measures the free unbound fractions of biogenic amines and their metabolites. The method eliminates sample cleanup/pre-concentration, and by using polarity switching to detect both protonated and deprotonated species allows us to measure 10 analytes (5 free biogenic monoamines and 5 metabolites) in a single chromatographic run.

2. Materials and methods

2.1. Chemicals and reagents

Epinephrine, norepinephrine, dopamine, serotonin, metanephrine, normetanephrine, 3-methoxytyramine, vanillylmandelic acid, 5-hydroxyindole-3-acetic acid, homovanillic acid, 3-methoxytyramine HCl, deuterated internal standards – (±)-epinephrine- d_6 , (±)-norepinephrine- d_6 , dopamine- d_4 , metanephrine- d_3 , (±)-normetanephrine- d_3 , (±)-4-hydroxy-3-methoxymandelic acid- d_3 , 4-hydroxy-3-methoxyphenyl- d_3 -acetic- d_2 acid, 5-hydroxyindole-4,6,7- d_3 -3-acetic- d_2 acid, and HCl were procured from Cerilliant, Round Rock, TX. 3-Methoxytyramine- d_4 and serotonin- $\alpha,\alpha,\beta,\beta$ - d_4 creatinine sulfate complex were obtained from CDN Isotopes Inc., Quebec, Canada. UHPLC-MS grade water, UHPLC-MS grade methanol, LC-MS grade formic acid, and Infinity™ Creatinine Liquid Stable Reagent were purchased from Thermo Fisher Scientific Inc., Waltham MA.

2.2. Preparation of standard solutions

Stock solutions of ten standards and labeled internal standards (IS) were prepared at various concentrations in Mass Spect Gold Urine (Golden West Biologicals, CA) and aliquots were stored at -80°C . Working solutions for a 9-point standard curve were prepared by appropriate dilutions of the stock solutions of calibrators and IS with 0.2% formic acid.

2.3. Study subjects and recruitment

Study participants with moderate-to-severe risk of cardiovascular disease were recruited from the University of Louisville Hospital and affiliated clinic system between October 2009 and December 2014. All participants gave written and informed consent prior to enrolling in the study. The Institutional Review Board (IRB number 09.0174) at the University of Louisville approved the study. Individuals with lung, liver, or kidney disease, coagulopathies, substance abuse, chronic cachexia and severe comorbidities, pregnant or lactating women, and prisoners were excluded from the study. Demographic information and medical history of study participants are provided in Table 1.

2.4. Urine sample collection and processing

Random catch urine was collected from each participant, aliquoted, and frozen at -80°C within 1 h after collection. Pre-stored urine samples of 255 non-smokers and 191 smokers were thawed on ice and vortex mixed. Samples (5 μL) were then mixed with 50 μL of IS mixture and 195 μL of 0.2% formic acid in a 2 mL amber UPLC sample vial. Vials were capped and vortex mixed.

2.5. UPLC-MS/MS analysis

2.5.1. Instrument setup and acquisition parameters—Urinary levels of *free* biogenic monoamines and their metabolites were measured by UPLC-MS/MS using a Xevo TQ-S micro quadrupole mass spectrometer with an ESI ionization source, interfaced with Waters Acquity Class-H UPLC equipped with a quaternary pump system (Waters, MA). The column compartment temperature was held at 40°C and the autosampler was set to 6°C . Samples (0.8 μL) were applied on UPLC and separated on an Acquity UPLC high strength silica (HSS) perfluorophenyl (PFP) (150 mm $\times$ 2.1 mm, 1.8 μm) column (Waters Inc., MA) with a binary gradient consisting of 0.2% formic acid (Solvent A) and methanol (Solvent B). The gradient started with 0.5% solvent B at a flow rate of 0.4 mL/min for 1 min and then ramped up to 95% solvent B at a flow rate of 0.35 mL/min over a period of 3 min. The gradient was then maintained at these conditions for 0.5 min before recycling back to 0.5% solvent B in 0.1 min, and then held at 0.5% solvent B at a flow rate of 0.4 mL/min for 5.4 min.

The MS/MS data was acquired in time-scheduled multiple reaction ion monitoring (MRM) mode using electrospray ionization. Polarity switching was used to detect both positive and negative ions in the same run. The following electrospray ionization inlet conditions were used: capillary 0.50 kV, cone 28 V, source temperature 150°C , desolvation temperature 600°C , cone gas flow 50 L/h, and desolvation gas flow 1000 L/h.

To reduce potential false positives, three multiple reaction monitoring (MRM) transitions were set up for each analyte: one for quantification, one for confirmation, and one for isotopically labeled internal standard (IS; Table 2). The integration was performed only on the peaks with retention time ± 0.03 min of IS, and with quantification/confirmation MRM peak area ratio within 30% of the corresponding calibrator.

A minimum of 15 data points were collected for each peak. TargetLynx quantification application manager software (Waters Inc., MA) was used for peak integration, calibration, and quantification. Analytes in the urine were quantified using peak area ratio based on 9-point standard curves, which were generated before and after analysis of urine samples. Data were normalized to creatinine (measured on a COBAS MIRA-plus chemical analyzer using Infinity™ creatinine reagent as described before [49–51]).

2.5.2. Accuracy, precision and recovery—Four levels of Quality Control (QC) samples were prepared by spiking known amount of analytes with urine. Within- and between-run accuracy and precision were evaluated by measuring these QC samples in three independent runs, with five replicates per QC level. To account for the pre-existing analytes in the QC urine matrix, we determined their levels through triplicate measurements before spike-in. After data collection, accuracy of the assay was calculated as the ratio of measured and the expected concentration of each analyte. Precision of the assay was calculated as reported by Chesher et al. [52]. Recovery was evaluated using three levels of QC samples.

2.5.3. Linearity and sensitivity—A weighted (1/X) standard curve was generated by plotting the ratio of the peak area of the analyte and the internal standard (Y) versus the calibrator concentration (X), and Pearson correlation coefficient (r) was calculated as the measurement of linearity. The lower limit of detection (LOD) and lower limit of quantification (LOQ) for each analyte was determined based on the standard deviation of the y-intercepts of regression lines and the slope of 8 calibration curves in different runs [53]. The results are summarized in Table 3.

2.5.4. Long-term stability—To assess long-term stability, both Lyphochek Quantitative Urine Controls (Bio-Rad Laboratories, Inc. CA, n = 24) and experimental urine samples (n = 30) were reanalyzed after storage at –80 °C for > 200 days subsequent to the first measurement. Accuracy was assessed by comparing the concentrations of the analyte after storage with that of the earlier run, and precision was calculated as CV (%).

2.5.5. Matrix effect—To assess the matrix effect a reference standard curve (without urine) was generated using a set of calibrators as described in Section 2.2. Slopes of standard curves constructed for the same set of calibrators spiked with urine (5 µL) obtained from 6 different subjects were compared with the reference standard curve.

2.6. Statistical analyses

Participant characteristics are expressed as n (%) for categorical variables (sex, race, cardiovascular history) and mean ($\pm$ SD) for continuous variables (age, BMI). The levels of urinary free biogenic monoamine/monoamine metabolites in smokers vs. non-smokers were compared using independent samples *t*-test. The levels of metabolites were log-transformed for normality and *p*-values represent log-transformed *t*-test results (Table 8). The association between the levels of urinary free biogenic monoamine/monoamine metabolites and smoking status was assessed using generalized linear models (Fig. 2). The data were right skewed and positive, therefore a gamma distribution with log link function was used to normalize the data. Models were adjusted for age, sex, race, BMI, and β -blocker use. For

each model, the percent change (and 95% confidence interval) was calculated for comparing smokers vs. non-smokers. Smoking status was confirmed using urinary cotinine levels, as described by Conklin et al. [55]. All statistical analyses were performed using SAS, version 9.4, software (SAS Institute, Inc., Cary, North Carolina) and GraphPad Prism, version 7 (GraphPad Software, La Jolla, California).

3. Results

3.1. Chromatography

Given the high sensitivity, dynamic range of 5–6 orders of magnitude of the mass spectrometer and the ability to perform ultrafast polarity switching, we reasoned that our instrumental setup would allow full coverage of biogenic monoamines and their metabolites in the concentrations found in urine by employing simple dilute-and-shoot approach coupled with the proper choice of chromatographic parameters. Indeed, as seen in Fig. 1, we found that all analytes of interest were retained ($k' > 1.50$) and efficiently separated on the PFP-modified C18 column in a 5-min chromatographic run (not including the reequilibration). Retention time, MRM transitions, and mass spectrometry parameters of the analytes and their stable isotopes are listed in Table 2. Importantly, UPLC with a 150 mm PFP column and 1.8 μm particle size afforded ample resolution of analytes, including EPI/NMN and 3-MT/MN – isobaric analyte pairs with identical MRM transitions (Table 2), and therefore, indistinguishable by MS/MS.

3.2. Method validation

3.2.1. Linearity and sensitivity—For our dilute-and-shoot methodological approach, we examined whether the linearity of the method is sufficient to cover a dynamic concentration range. As seen in Table 3, the R^2 determined for all analytes was > 0.995 . The LOD and LOQ values for all analytes are comparable with other LC-MS/MS assays for free catecholamines and metanephrines [13,56,57].

3.2.2. Accuracy, precision and recovery—As listed in Table 4, the within- and between run accuracy of all analytes were $100 \pm 16\%$. The within- and between run precision values, expressed as coefficient of variation (CV), were $< 15\%$ for all analytes, with the exception of 5-HT at LOQ level (17.3%). The recovery of all analytes was determined by spiking the urine matrix with three different concentrations of the standards (low, middle and high spike), listed in Table 5. The recovery was within the range of 92–113% at all spike levels ($SD \pm 16\%$). Collectively, these results demonstrate excellent precision and accuracy for the determination of all the analytes in a small volume of urine samples.

3.2.3. Long-term stability—As shown in Table 6, the concentration values of analytes after storage for > 200 days at -80°C were comparable to earlier measurements. The accuracy was within 93–111% range. These data suggest that long term storage of human urine samples does not affect the levels of free biogenic amines and their metabolites.

3.2.4. Matrix effect—To examine the impact of matrix effect on quantification, we compared slopes of standard curves of samples containing urine with reference standard curve [54]. As shown in Table 7, the addition of urine matrix had small influence (< 5%) on the slope of the standard curves, with the exception of HVA, whose coefficient of variation was 7.2%.

3.3. Application of the assay to measure free biogenic monoamines and their metabolites in the urine of smokers

After initial development and testing, we applied our method to identify and quantify *free* biogenic monoamines and their metabolites in spot urine samples collected from 255 non-smokers and 191 smokers. Demographic characteristics of study participants are tabulated in Table 1. Generalized linear models adjusted for confounding factors, including age, sex, race, BMI, and β -blocker use were used to test the association between *free* biogenic monoamines and their metabolites with smoking status. These data show that under the experimental condition used, there is minimal effect of matrix (human urine) on the resolution of various analytes, and that the carryover effect is negligible (< 1%). When normalized to urinary creatinine, the levels of urinary catecholamines, 5-HT, metanephrines, VMA, and HVA measured by this procedure were in the range reported in previous studies [1,4,18,41,58]. The levels of urinary free biogenic monoamines, and metanephrines were significantly higher in smokers than in non-smokers (Fig. 2). These observations are in agreement with previous work showing that the levels of free biogenic monoamines in bio-fluids and tissues are higher in smokers than in non-smokers [17–21]. In contrast, the urinary levels of stable metabolites of catecholamines – VMA, HVA were significantly lower in smokers than in non-smokers. As such, we were unable to find literature reporting the urinary levels of these metabolites in tobacco product users, especially in comparison to nontobacco users. No difference in the urinary levels of 5-HIAA was found between smokers and non-smokers.

4. Discussion

We developed a simple, comprehensive, robust, and high-throughput method for quantitative analyses of urinary free biogenic monoamines and their metabolites. Our dilute-and-shoot approach eliminates sample pre-treatment and extraction, and minimizes sample processing. Polarity switching used in our method enabled the analyses of both acidic and basic analytes in the same chromatographic run, which represents a distinct advantage over current methods. Moreover, the use of chromatographic separation on 150 mm PHP column allowed for ample retention and baseline resolution of all analytes, including difficult to resolve isobars and difficult to retain polar catecholamines (e.g. NE). We believe that our UPLC/MS/MS analyses afford unprecedented sensitivity, dynamic range, and reliable separation of 10 different analytes in a single 5-min analytical run. Finally, we demonstrate the efficacy and the applicability of the newly developed method by measuring urinary levels of free biogenic amines and their metabolites in a large cohort of smokers and non-smokers.

Biogenic monoamines and their metabolites are often measured for the diagnosis of certain types of tumors, such as neuroblastoma [1–5] and pheochromocytoma [6–8]. Previous work

has shown that increased levels of catecholamines are also associated with smoking [18,19,59–61], cardiovascular disease [14,17,62] and neurological disorders [10,12,28]. Often catecholamines are measured by HPLC with electrochemical detection [18,63,64], a method that was established over 30 years ago [18,44]. These assays have a number of drawbacks including tedious sample extraction and poor recovery, low sensitivity, poor resolution of analytes, and potential interferences from drugs and other contaminants, which may lead to overestimation of analytes as well as false positives [6,58]. Moreover, these assays require independent chromatographic analyses of basic compounds - catecholamines [32,40,41] and metanephrines [24,42], and their acidic metabolites (VMA, HVA and 5-HIAA) [25,43]. Hence, such procedures are time consuming, expensive, and require relatively large amount of specimen.

Our newly developed UPLC-MS/MS method eliminates most of these shortcomings. The use of UPLC with a 150 mm PFP column (1.8 μ m particle size) enables the retention and resolution of 10 analytes including isobars (e.g. EPI/NMN and 3-MT/MN), which are typically difficult to resolve [40], and polar catecholamines, such as NE, which are usually difficult to retain on the column. The MRM based detection and quantification using isotopically labeled internal standards coupled with fast polarity switching allowed concurrent detection of basic and acidic species with linear response over a wide range of physiological concentrations. Further gains in sample throughput were obtained by replacing the costly and time consuming solid phase extraction and/or derivatization step employed in previous methods [24,25,32,40–43,47] with a simple dilute-and-shoot approach. This avoided possible analyte losses from solid phase extraction or derivatization. Moreover, good analyte recovery (between 92.6% and 113.1%), a wide dynamic range, and minimal matrix effects make the method highly suitable for the analysis of human urine samples from a large cohort and clinical studies.

To establish the applicability of our newly developed method, we measured the levels of free biogenic monoamines and their metabolites in the urine of 255 non-smokers and 191 smokers. The observed increase in free catecholamines – EPI, NE, and DA; and 5-HT levels in smokers in our study is in agreement with previous studies reporting that the consumption of tobacco products increases the production of free catecholamines [59,60,65–69]. In addition to free biogenic monoamines, we could also measure the urinary levels of metanephrines and their downstream metabolites. These measurements provide novel data, as the urinary levels of free biogenic monoamines in smokers have not been reported before. We also found that smokers have significantly higher levels of metanephrines, which are derived from the activation of catechol-*O*-methyltransferase. Interestingly, we also observed that catecholamine metabolites synthesized through the MAO pathway – HVA and VMA, were significantly lower in smokers than in non-smokers. This may be due to the inhibition of MAO by constituents of tobacco smoke such as norharman, benzoquinone, and naphthylamine [70–73].

Although, in this study we only measured the levels of free biogenic monoamines and their metabolites in smokers, our method has extensive applications. For example, urinary analysis of free biogenic monoamines and their metabolites could be invaluable in the differential diagnosis of tumors [1–3,5–7,9]. Currently, for such measurements, one has to

first measure free catecholamine levels in the urine and then use another assay to quantify metabolites of biogenic monoamines [2,6,7]. Overall, current approaches are cumbersome, time consuming and costly. In comparison, our method can measure all these analytes in a single 5 min run. Because of its simplicity and robustness, our method will also be useful in studying changes in neurotransmitter metabolism per se. Comprehensive analyses on free biogenic monoamines and their metabolites also opens up new avenues to examine how different physiological and pathological conditions affect biogenic amine production and metabolism and how these processes are affected by different drugs and xenobiotics.

Acknowledgments

Funding

This work was supported in parts by NIH grants U54 HL120163, P30 GM127607, R01 ES029846 and P42 ES023716.

Abbreviations:

EPI	epinephrine
NE	norepinephrine
DE	dopamine
MN	metanephrine
NMN	normetanephrine
3-MT	3-methoxytyramine
5-HT	serotonin
5-HIAA	5-hydroxyindole-3-acetic acid
VMA	vanillylmandelic acid
HVA	homovanillic acid
MOPEGAL	3-methoxy-4-hydroxyphenylglycolaldehyde
HMA	4-hydroxy-3-methoxyphenylacetaldehyde
5-HIAL	5-hydroxyindole-3-acetaldehyde
COMT	catechol- <i>O</i> -methyltransferase
MAO	L-monoamine oxidase
UPLC-MS/MS	ultrahigh performance liquid chromatography with tandem mass spectrometry

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Fig. 1. MRM LC-MS/MS chromatograms of biogenic monoamines and their metabolites. A. Chromatograms of reagent analytes. B. Representative chromatograms of analytes in human urine samples. Epinephrine (EPI), norepinephrine (NE), dopamine (DA), metanephrine (MN), normetanephrine (NMN) 3-methoxytyramine (3-MT), serotonin (5-HT), 5-Hydroxyindole-3-acetic acid (5-HIAA), vanillylmandelic acid (VMA) and homovanillic acid (HVA).

Fig. 2. Percent change in the urinary *free* biogenic monoamines and their metabolites in smokers vs. non-smokers. Analysis consisted of 446 participants (255 non-smokers and 191 smokers). Generalized linear models were used to examine the association between urinary catecholamine levels and smoking status. Models were adjusted for age, sex, race, BMI, and β -blocker use. Smoking status was determined by measuring urinary cotinine levels. Values represent percent change and 95% confidence interval.

Scheme 1.

Phase I metabolites of biogenic monoamines. Epinephrine (EPI), norepinephrine (NE), dopamine (DE), metanephrine (MN), normetanephrine (NMN) 3-methoxytyramine (3-MT), serotonin (5-HT), 5-hydroxyindole-3-acetic acid (5-HIAA), vanillylmandelic acid (VMA), homovanillic acid (HVA), 3-methoxy-4-hydroxyphenylglycolaldehyde (MOPEGAL), 4-hydroxy-3-methoxyphenylacetaldehyde (HMA) and 5-hydroxyindole-3-acetaldehyde (5-HIAL). The two enzymes are catechol-*O*-methyltransferase (COMT) and L-monoamine oxidase (MAO).

Table 1

Demographic summary and recruitment information for study participants.

Variables	Total (N = 446)	Non-smokers (N = 255)	Smokers (N = 191)
Age	51.3 ± 10.9	51.9 ± 11.9	50.5 ± 9.2
Sex			
Male	226 (51%)	122 (48%)	104 (54%)
Female	220 (49%)	133 (52%)	87 (46%)
Race			
African American	169 (38%)	92 (36%)	77 (40%)
Caucasian	256 (57%)	148 (58%)	108 (57%)
Other	21 (5%)	15 (6%)	6 (3%)
BMI	32.8 ± 8.1	33.7 ± 8.1	31.6 ± 7.9
Cardiovascular history			
Hypertension	325 (73%)	181 (71%)	144 (76%)
Hyperlipidemia	268 (61%)	152 (60%)	116 (62%)
Diabetes	139 (31%)	89 (35%)	50 (26%)
Myocardial infarction	139 (31%)	68 (27%)	71 (37%)
Heart failure	82 (18%)	41 (16%)	41 (21%)

Abbreviations: Body mass index (BMI).

Table 2
 MRM transitions, MS parameters, and retention time of analytes and their deuterated analogs.

Analytes	Ionization mode	MRM type	MRM transitions	Cone voltage (V)	Collision (eV)	Dwell time (s)	Retention time (min)
EPI	ESI ⁺	Quan	18- > 166	22	14	0.2	1.79
EPI-d ₆	ESI ⁺	Conf	184- > 107	22	22	0.025	
		IS	190- > 172	22	14	0.1	
NE	ESI ⁺	Quan	152- > 107	38	18	0.075	1.25
		Conf	152- > 79	38	20	0.02	
NE-d ₆	ESI ⁺	IS	158- > 139	38	8	0.025	
		Quan	154- > 91	27	21	0.1	2.13
DA	ESI ⁺	Conf	154- > 119	27	16	0.025	
		IS	158- > 121	27	16	0.025	
5-HT	ESI ⁺	Quan	160- > 115	56	20	0.04	3.88
		Conf	160- > 132	56	14	0.01	
5-HT-d ₄	ESI ⁺	IS	164- > 136	56	14	0.02	
		Quan	180- > 165	48	14	0.045	3.48
MN	ESI ⁺	Conf	180- > 148	48	14	0.015	
		IS	183- > 151	48	14	0.025	
NMN	ESI ⁺	Quan	166- > 134	40	14	0.1	2.10
		Conf	166- > 121	40	14	0.025	
NMN-d ₃	ESI ⁺	IS	169- > 137	40	14	0.025	
		Quan	168- > 91	26	17	0.08	3.80
3-MT	ESI ⁺	Conf	168- > 119	26	23	0.025	
		IS	172- > 123	26	17	0.025	
3-MT-d ₄	ESI ⁺	Quan	197- > 138	36	12	0.1	3.36
		Conf	197- > 137	36	20	0.025	
VMA	ESI ⁻	IS	200- > 141	36	12	0.05	
		Quan	227- > 181	10	7	0.04	4.45
HVA	ESI ⁻	Conf	181- > 137	25	7	0.015	
		IS	186- > 142	25	8	0.01	

Analytes	Ionization mode	MRM type	MRM transitions	Cone voltage (V)	Collision (eV)	Dwell time (s)	Retention time (min)
5-HIAA	ESI ⁺	Quan	192–> 146	26	12	0.05	4.29
		Conf	192–> 91	26	32	0.01	
5-HIAA-d ₅	ESI ⁺	IS	197–> 150	26	12	0.01	

Abbreviations: epinephrine (EPI), norepinephrine (NE), dopamine (DA), serotonin (5-HT), metanephrine (MN), normetanephrine (NMN), 3-methoxytyramine (3-MT), vanillylmandelic acid (VMA), homovanillic acid (HVA), 5-hydroxyindole-3-acetic acid (5-HIAA).

Table 3

Standard curve range, linearity, lower limit of detection (LOD), and lower limit of quantification (LOQ) of 10 analytes.

Analyte	Linearity range	Standard curve linearity (r^2)	LOD	LOQ
	(ng/mL in urine)		(ng/mL in urine)	(ng/mL in urine)
EPI	2–400	0.998940	0.65	1.96
NE	2–400	0.999658	0.67	2.03
DA	10–4000	0.999636	2.28	6.90
5-HT	5–2000	0.999286	1.44	4.38
MN	2.5–1000	0.999489	2.35	7.13
NMN	2.5–1000	0.999440	1.56	4.74
3-MT	2.5–1000	0.999701	0.91	2.95
VMA	250–100,000	0.999059	117	355
HVA	250–100,000	0.997400	421	1276
5-HIAA	250–100,000	0.999427	82.9	251

Abbreviations: limit of detection (LOD), limit of quantification (LOQ), epinephrine (EPI), norepinephrine (NE), dopamine (DA), serotonin (5-HT), metanephrine (MN), normetanephrine (NMN), 3-methoxytyramine (3-MT), vanillylmandelic acid (VMA), homovanillic acid (HVA), 5-hydroxyindole-3-acetic acid (5-HIAA).

Table 4

Determination of within- and between run accuracy and precision (based on 3 independent runs with 5 replicates at each level).

Analyte	Level (ng/mL)	Within-run accuracy (%)	Between run accuracy (%)	Within-run precision CV (%)	Between run precision (%)
EPI	2.58	101.2	99.4	7.2	12.3
	5.58	101.9	104.0	6.5	6.3
	50.6	100.1	99.5	2.6	2.7
	201	98.9	99.5	1.7	3.2
NE	2.19	99.4	101.9	11.5	10.3
	6.19	95.6	91.0	10.1	9.1
	51.2	98.4	99.2	4.5	4.3
	201	99.0	98.6	1.9	1.9
DA	17.0	92.8	94.0	3.9	5.4
	27.0	97.4	98.0	4.4	4.1
	267	100.8	101.2	1.6	1.7
	2017	99.6	99.4	2.5	2.4
5-HT	4.45	95.5	97.3	14.7	17.3
	9.45	104.9	102.8	7.2	8.3
	129	100.8	100.9	3.1	3.0
	1004	98.5	98.7	2.5	2.4
MN	5.79	98.4	97.4	12.9	12.1
	13.3	98.6	95.7	6.5	6.2
	126	99.3	99.4	2.0	2.4
	501	98.8	98.7	1.8	1.9
NMN	5.91	97.3	95.3	11.5	12.7
	13.4	97.3	98.0	4.6	4.8
	126	100.1	99.5	2.4	2.2
	501	100.6	101.5	2.5	2.4
3-MT	3.66	104.7	106.3	8.9	8.6
	6.16	100.6	100.7	5.8	5.2
	63.7	101.1	101.0	1.5	1.7
	501	99.8	100.5	2.6	3.5
VMA	186	90.5	91.5	4.3	7.0
	436	95.8	95.3	5.6	6.6
	6436	99.5	99.0	2.9	2.7
	50,186	99.2	98.0	4.7	4.5
HVA	1264	115.9	114.4	9.3	13.1
	2514	113.0	111.9	10.0	12.5
	12,514	100.1	100.6	8.1	8.1
	50,014	102.3	104.6	8.1	8.3
5-HIAA	375	112.3	114.0	3.1	3.9

Analyte	Level (ng/mL)	Within-run accuracy (%)	Between run accuracy (%)	Within-run precision CV (%)	Between run precision (%)
	625	108.9	108.6	4.3	5.0
	6375	102.1	102.4	2.5	2.9
	50,125	95.5	95.7	2.4	3.0

Abbreviations: coefficient of variation (CV), epinephrine (EPI), norepinephrine (NE), dopamine (DA), serotonin (5-HT), metanephrine (MN), normetanephrine (NMN), 3-methoxytyramine (3-MT), vanillylmandelic acid (VMA), homovanillic acid (HVA), 5-hydroxyindole-3-acetic acid (5-HIAA).

Table 5

Analyte recovery analyses of human urinary samples (n = 15).

Analyte	Background (ng/mL)	Standard added (ng/mL)	Recovery (%)	SD (%)
EPI	0.58	5	102.1	7.2
		50	100.1	2.7
		200	98.9	2.9
NE	1.19	5	94.6	11.1
		50	98.4	4.4
		200	99.0	1.9
DA	16.99	10	93.0	11.1
		250	100.8	1.8
		2000	99.6	2.4
5-HT	4.45	5	109.2	15.8
		125	100.8	3.2
		1000	98.5	2.4
MN	0.79	12.5	98.5	6.6
		125	99.3	2.3
		500	98.8	1.8
NMN	0.91	12.5	97.0	5.0
		125	100.1	2.3
		500	100.6	2.4
3-MT	1.16	5	100.8	6.6
		62.5	101.2	1.7
		500	99.8	3.3
VMA	186.15	250	92.6	10.7
		6250	99.5	2.8
		50,000	99.2	4.5
HVA	13.93	2500	113.1	13.5
		12,500	100.1	8.1
		50,000	102.3	8.4
5-HIAA	124.51	500	111.1	6.5
		6250	102.2	2.9
		50,000	95.5	2.8

Abbreviations: standard deviation (SD), epinephrine (EPI), norepinephrine (NE), dopamine (DA), serotonin (5-HT), metanephrine (MN), normetanephrine (NMN), 3-methoxytyramine (3-MT), vanillylmandelic acid (VMA), homovanillic acid (HVA), 5-hydroxyindole-3-acetic acid (5-HIAA).

Table 6

Long-term stability investigations in human urine samples ($n = 30$) and Lyphochek Quantitative Urine Controls (Level 1 Normal and Level 2 Abnormal, $n = 24$ for both).

Analyte	Level 1 normal		Level 2 abnormal		Human urine samples	
	Accuracy (%)	Precision CV (%)	Accuracy (%)	Precision CV (%)	Accuracy (%)	Precision CV (%)
EPI	110.1%	5.1%	99.7%	3.7%	107.3%	13.5%
NE	96.7%	4.8%	101.3%	3.2%	97.5%	5.4%
DA	100.2%	3.2%	101.6%	2.5%	101.0%	5.7%
5-HT	94.9%	9.2%	97.9%	7.3%	102.5%	10.2%
MN	100.4%	2.8%	99.5%	2.1%	108.6%	9.6%
NMN	102.0%	2.4%	101.7%	2.4%	96.4%	7.1%
3-MT	94.4%	3.3%	99.5%	1.9%	102.1%	4.4%
VMA	97.3%	8.0%	99.1%	5.5%	93.0%	5.9%
HVA	95.9%	9.1%	97.2%	4.2%	98.6%	19.7%
5-HIAA	98.7%	2.1%	99.6%	2.7%	102.3%	4.6%

The samples were stored in -80°C for > 200 days.

Abbreviations: coefficient of variation (CV), epinephrine (EPI), norepinephrine (NE), dopamine (DA), serotonin (5-HT), metanephrine (MN), normetanephrine (NMN), 3-methoxytyramine (3-MT), vanillylmandelic acid (VMA), homovanillic acid (HVA), 5-hydroxyindole-3-acetic acid (5-HIAA).

Table 7

Matrix effect determination.

Analytes	Slope difference average (%)	Slope CV (%)
EPI	3.1%	1.3%
NE	1.3%	2.6%
DA	1.2%	1.5%
5-HT	4.4%	3.2%
MN	0.8%	1.6%
NMN	3.1%	2.5%
3-MT	2.7%	2.8%
VMA	1.1%	1.9%
HVA	4.0%	7.2%
5-HIAA	4.9%	3.1%

Abbreviations: coefficient of variation (CV), epinephrine (EPI), norepinephrine (NE), dopamine (DA), serotonin (5-HT), metanephrine (MN), normetanephrine (NMN), 3-methoxytyramine (3-MT), vanillylmandelic acid (VMA), homovanillic acid (HVA), 5-hydroxyindole-3-acetic acid (5-HIAA).

Table 8

Urinary levels of free biogenic amines and their metabolites grouped by smoking status. Values are mean $\pm$ SD. **P*-values represent results from unpaired student's *t*-test on log-transformed data (for normality).

Analyte	Smokers, n = 191 Concentration (ng/mg creatinine)	Non-smokers, n = 255 Concentration (ng/mg creatinine)	p-Value*
EPI	7.7 $\pm$ 9.6	5.8 $\pm$ 4.3	0.020
NE	44.6 $\pm$ 27.5	37.5 $\pm$ 19.3	0.002
DA	188.3 $\pm$ 84.7	167.7 $\pm$ 74.3	0.015
5-HT	83.1 $\pm$ 41.7	74.9 $\pm$ 43.3	0.064
MN	16.9 $\pm$ 9.1	13.0 $\pm$ 5.9	< 0.001
NMN	30.6 $\pm$ 16.0	24.6 $\pm$ 10.4	< 0.001
3-MT	50.5 $\pm$ 21.8	39.6 $\pm$ 14.3	< 0.001
HVA	3071.8 $\pm$ 1449.2	3455.5 $\pm$ 1783.7	0.009
VMA	2869.1 $\pm$ 1262.6	3210.8 $\pm$ 1127.8	< 0.001
5-HIAA	2909.6 $\pm$ 1789.9	3070.9 $\pm$ 2548.3	0.986

Abbreviations: epinephrine (EPI), norepinephrine (NE), dopamine (DA), serotonin (5-HT), metanephrine (MN), normetanephrine (NMN), 3-methoxytyramine (3-MT), vanillylmandelic acid (VMA), homovanillic acid (HVA), 5-hydroxyindole-3-acetic acid (5-HIAA).