SUPPORTING INFORMATION

IN MATRIX DERIVATIZATION IN COMBINATION WITH LC-MS/MS RESULTS IN ULTRA-SENSITIVE QUANTIFICATION OF PLASMA FREE METANEPHRINES AND CATECHOLAMINES

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Table S-1. Transitions, cone voltage and collision energy for all analytes.

Analyte	Precursor (m/z)	Product (m/z)	Cone (V)	Collision energy (eV)
L-DOPA-Quan	366.1	152.05	18	35
L-DOPA-Qual	366.1	208.1	18	22
L-DOPA-Quan-d3	369.1	155.05	18	35
L-DOPA-Qual-d3	369.1	211.1	18	22
DA-Quan	322.1	137.05	18	30
DA-Qual	322.1	266.1	18	12
DA-Quan-d4	326.1	141.05	18	30
DA-Qual-d4	326.1	270.1	18	12
NE-Quan	320.1	152.05	20	28
NE-Qual	320.1	264.1	20	11
NE-Quan-d6	326.1	158.05	20	28
NE-Qual-d6	326.1	270.1	20	11
E-Quan	334.1	222.1	28	22
E-Qual	334.1	278.15	28	14
E-Quan-d3	337.1	225.1	28	22
E-Qual-d3	337.1	281.15	28	14
3-MT-Quan	280.1	151.05	22	23
3-MT-Qual	280.1	224.1	22	12
3-MT-Quan-d4	284.1	155.05	22	23
3-MT-Qual-d4	284.1	228.1	22	12
NMN-Quan	278.15	166.05	20	18
NMN-Qual	278.15	222.1	20	12
NMN-Quan-d3	281.15	169.05	20	18
NMN-Qual-d3	281.15	225.1	20	12
MN-Quan	292.1	180.05	28	22
MN-Qual	292.1	236.1	28	13
MN-Quan-d3	295.1	183.05	28	22
MN-Qual-d3	295.1	239.1	28	13

Abbreviations: Quan, quantifier; Qual, qualifier; DA, dopamine; NE, norepinephrine; E, epinephrine; 3-MT, 3-methoxytyramine; NMN, normetanephrine; MN, metanephrine; m/z, mass-over-charge; V, voltage; eV, electronvolt.

Table S-2. Result of the derivatization optimization experiment. Results were normalized to the result of undiluted propionic anhydride. The mean internal standard area response for n = 6 samples was used.

Analyte	Undiluted (%)	1:1 (%)	1:4 (%)	1:10 (%)
L-DOPA	100	123	154	149
Dopamine	100	103	115	105
Norepinephrine	100	111	119	103
Epinephrine	100	106	113	99
3-Methoxytyramine	100	106	118	112
Normetanephrine	100	121	126	125
Metanephrine	100	106	117	106

Table S-3. Mean recovery results (SD between parentheses).

Recovery in %		
	Spiked amounts (nmol/L)	Recovery (SD), %
L-DOPA	14.4	101 (8)
	43.1	97 (4)
	344	101 (3)
Dopamine	0.15	104 (8)
	0.45	100 (4)
	3.57	101 (2)
Norepinephrine	0.66	105 (7)
	1.97	95 (4)
	15.7	99 (3)
Epinephrine	0.19	99 (7)
	0.58	98 (2.5)
	4.63	100 (2)
3-Methoxytyramine	0.14	97 (5)
	0.42	97 (3)
	3.36	100 (2)
Normetanephrine	0.55	97 (7)
	1.64	98 (5)
	13.1	99 (1)
Metanephrine	0.21	95 (9)
	0.64	96 (6)
	5.1	99 (3)

Table S-4. Results for RCPAQAP samples.

		3-Methoxytyramine (pmol/L)			Normetanephrine (pmol/L)			Metanephrine (pmol/L)		
Month	ID	Target	Result	Error (%)	Target	Result	Error (%)	Target	Result	Error (%)
January	23-01	960	1000	4.0	2200	2220	0.9	1320	1380	4.3
	23-02	750	760	1.3	1790	1850	3.2	1060	1070	0.9
February	23-03	130	130	0	460	440	-4.5	290	310	6.5
	23-04	806	770	-4.7	1873	1780	-5.2	1098	1030	-6.6
March	23-05	1180	1200	1.7	2620	2720	3.7	1590	1640	3.0
	23-06	130	120	-8.3	460	410	-12	290	280	-3.6

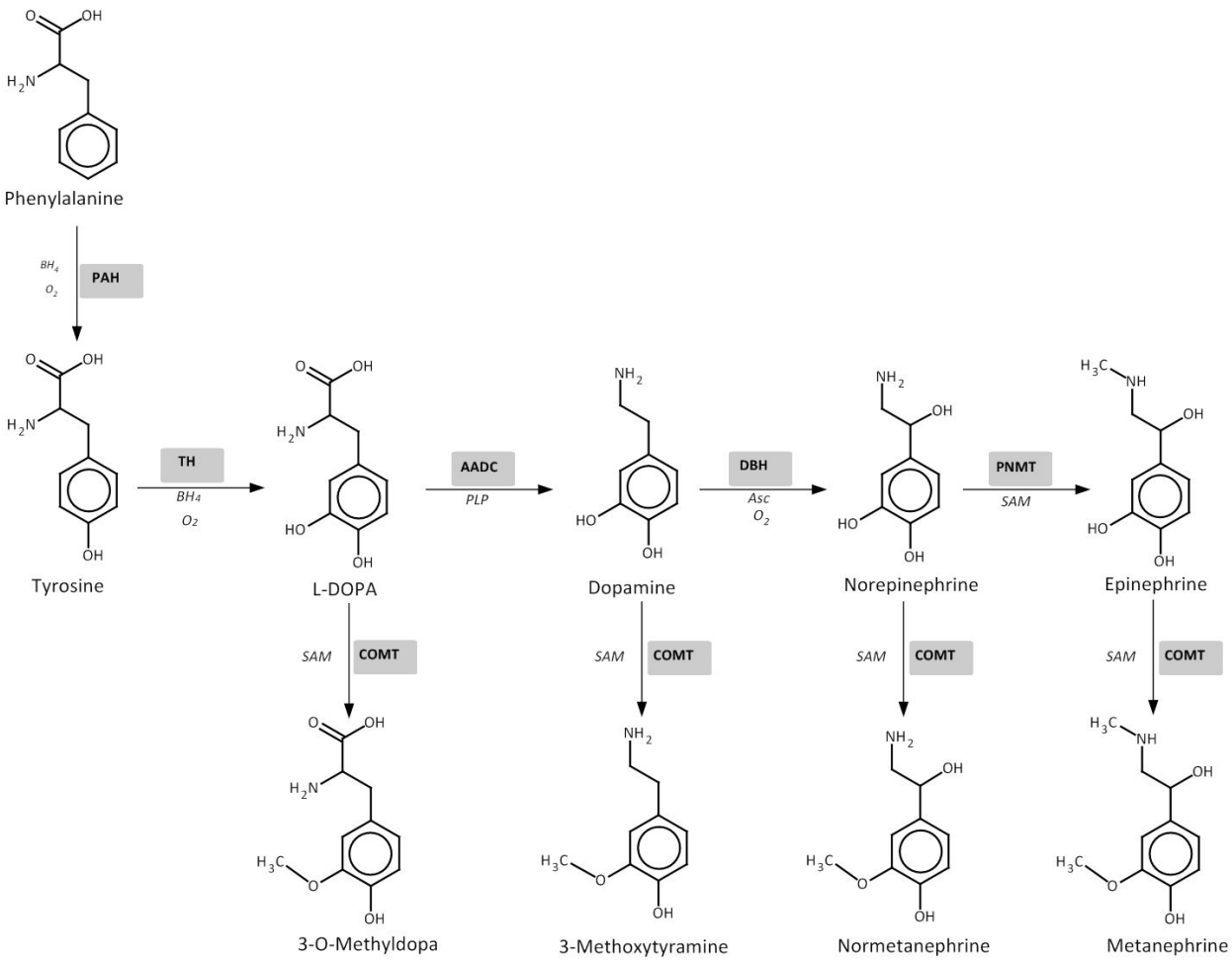
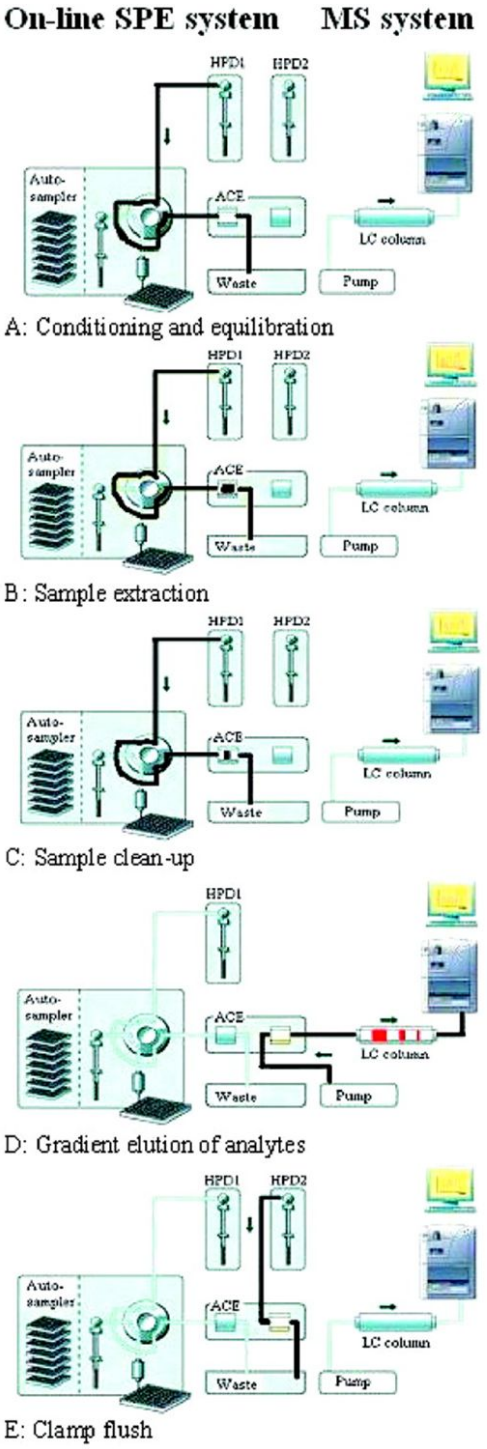


Figure S-1. Chemical structures of precursors, catecholamines, and their O-methylated metabolites, including pathways of metabolism. Abbreviations: PAH, phenylalanine hydroxylase; TH, tyrosine hydroxylase; BH4, tetrahydrobiopterin; AADC, aromatic L-amino acid decarboxylase; PLP, pyridoxal-5-phosphate; DBH, dopamine beta hydroxylase; Asc, ascorbic acid; PNMT, phenylethanolamine-N-methyltransferase; COMT, catechol-O-methyltransferase; SAM, S-adenosylmethionine.



A: Conditioning and equilibration

A1: Conditioning	Acetontrile , (0.5 mL at 5 mL/min)
A2: Conditioning	Magic mix, (0.5 mL at 5 mL/min)
A3: Equilibration	Water, (0.5mL at 5mL/min)

B: Sample extraction

B1: Injection	100 µL of sample
B2: Extraction	Sample loading using 0.1% formic acid in water, (0.5 mL at 2 mL/min)

C: Sample clean-up

C1: Wash	20% MeOH in 4 mM ammonium acetate (NH4Ac) + 0.1% ammonia, (0.5 mL, 2 mL/min)
C2: Wash	20% MeOH in 4 mM NH4Ac + 0.1% formic acid, (0.5 mL, 2 mL/min)
C3: Wash	20% acetonitrile in 4 mM NH4Ac + 0.1% formic acid, (0.25 mL, 2 mL/min)

D: Gradient elution of analytes

D1: Elution	Elution of analytes from cartridge to LC column using LC gradient (1.5 min)

E: Clamp flush

E1: Clamp flush	40% acetonitrile in water + 0.2% formic acid (0.5 mL, 5 mL/min)
E2: Clamp flush	Magix mix (0.5mL, 5 mL/min)
E3: Clamp flush	Acetonitrile (0.5 mL, 5 mL/min)
E4: Clamp flush	Water (0.5 mL, 5 mL/min)

Figure S-2. Scheme of the online SPE procedure and elution with the gradient pumps. Magic mix consists of a mixture methanol/isopropanol/acetonitrile/water (1:1:1:1) and 0.2% formic acid. Figure adapted from De Jong et al. 15.

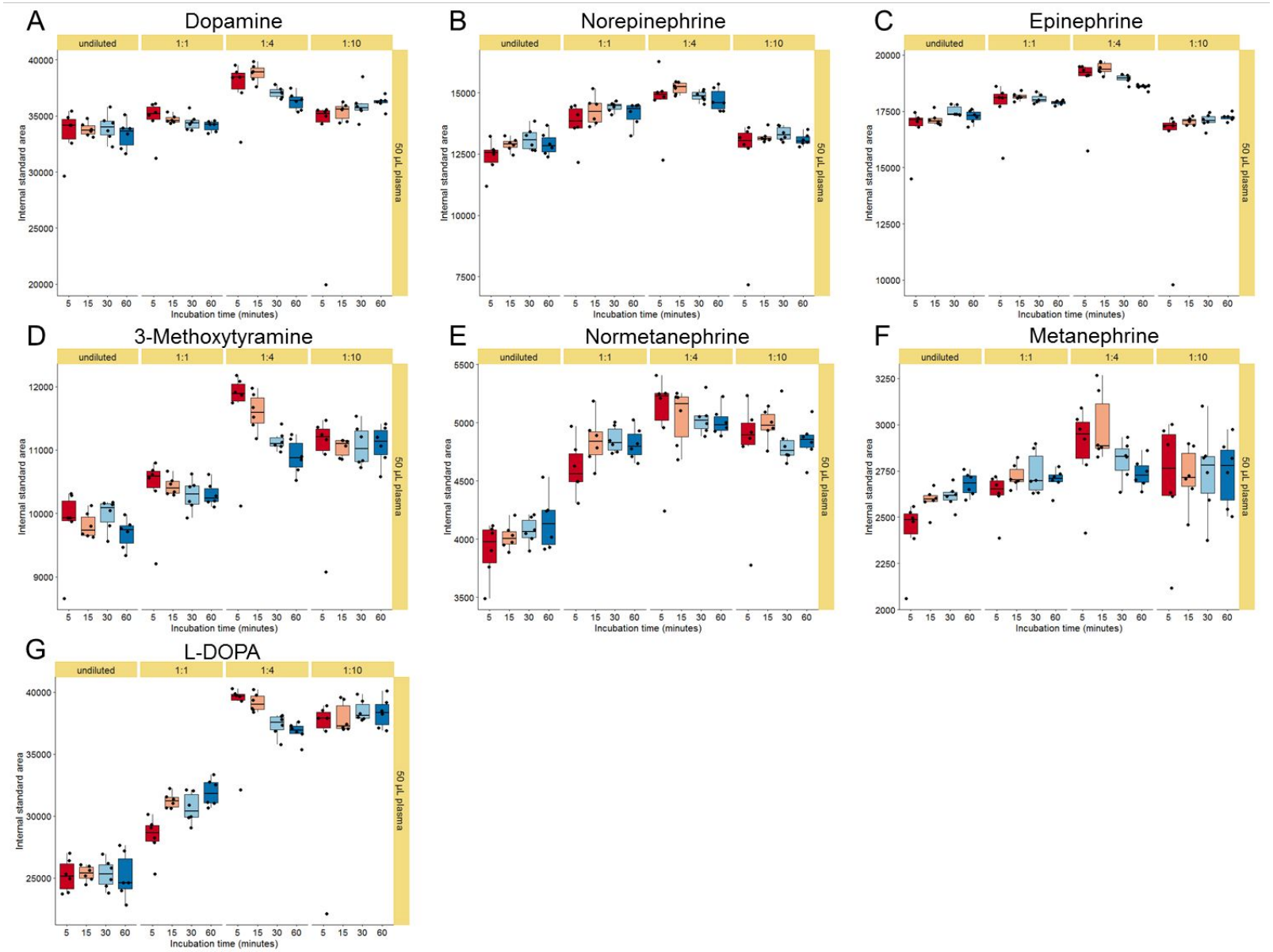

A
Dopamine
B
Norepinephrine
C
Epinephrine
D
3-Methoxytyramine
E
Normetanephrine
F
Metanephrine
G
L-DOPA
undiluted
1:1
1:4
1:10
50 µL plasma
Internal standard area
Incubation time (minutes)
5 15 30 60

Figure S-3. Effect of incubation time of the derivatization reaction on the internal standard peak area response for the three different catecholamines and metanephrines. Internal standard peak area is shown on the y-axis and the incubation time on the x-axis. (A) Results for dopamine-D4 for 50 µL plasma. (B) Results for norepinephrine-D6 for 50 µL plasma. (C) Results for epinephrine-D3 for 50 µL plasma. (D) Results for 3-methoxytyramine-D4 for 50 µL plasma. (E) Results for nor-metanephrine-D3 for 50 µL plasma. (F) Results for metanephrine-D3 for 50 µL plasma. (G) Results for L-DOPA-d3 for 50 µL plasma.

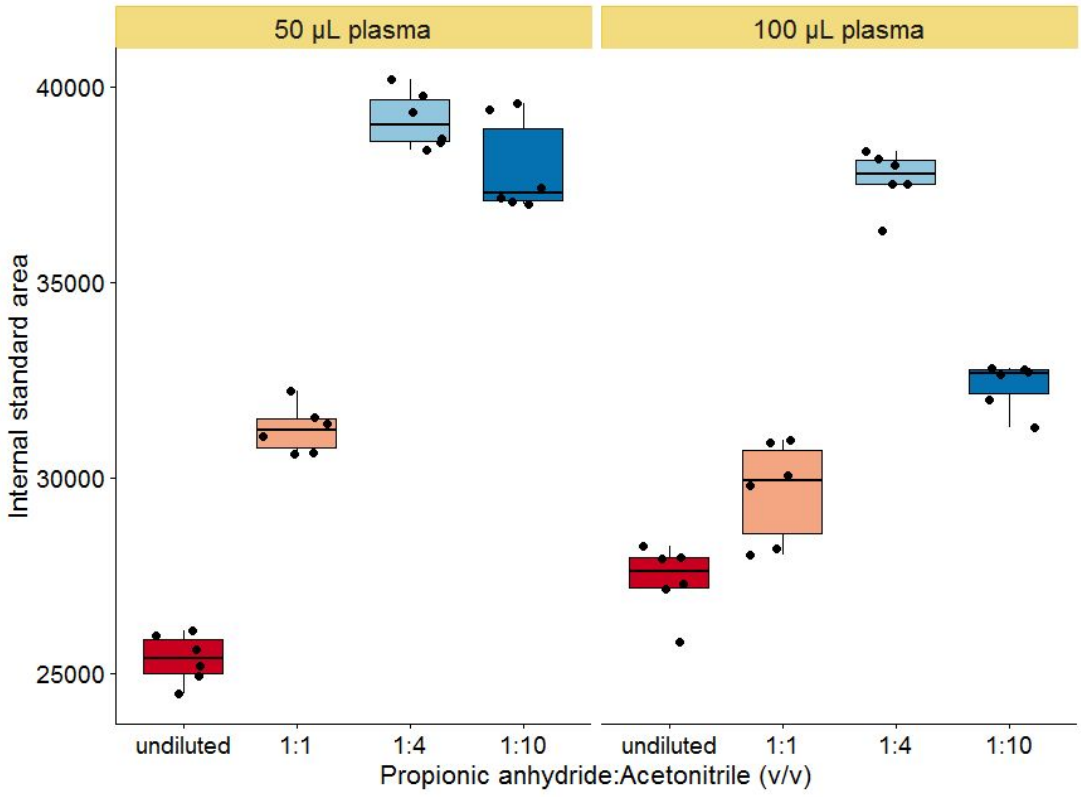


Figure S-4. Effect of different derivatization reaction conditions on the internal standard peak area response for L-DOPA. Internal standard peak area is shown on the y-axis and the ratio of propionic anhydride to acetonitrile (v/v) on the x-axis. Results for L-DOPA-D3 for 50 µL and 100µL plasma.

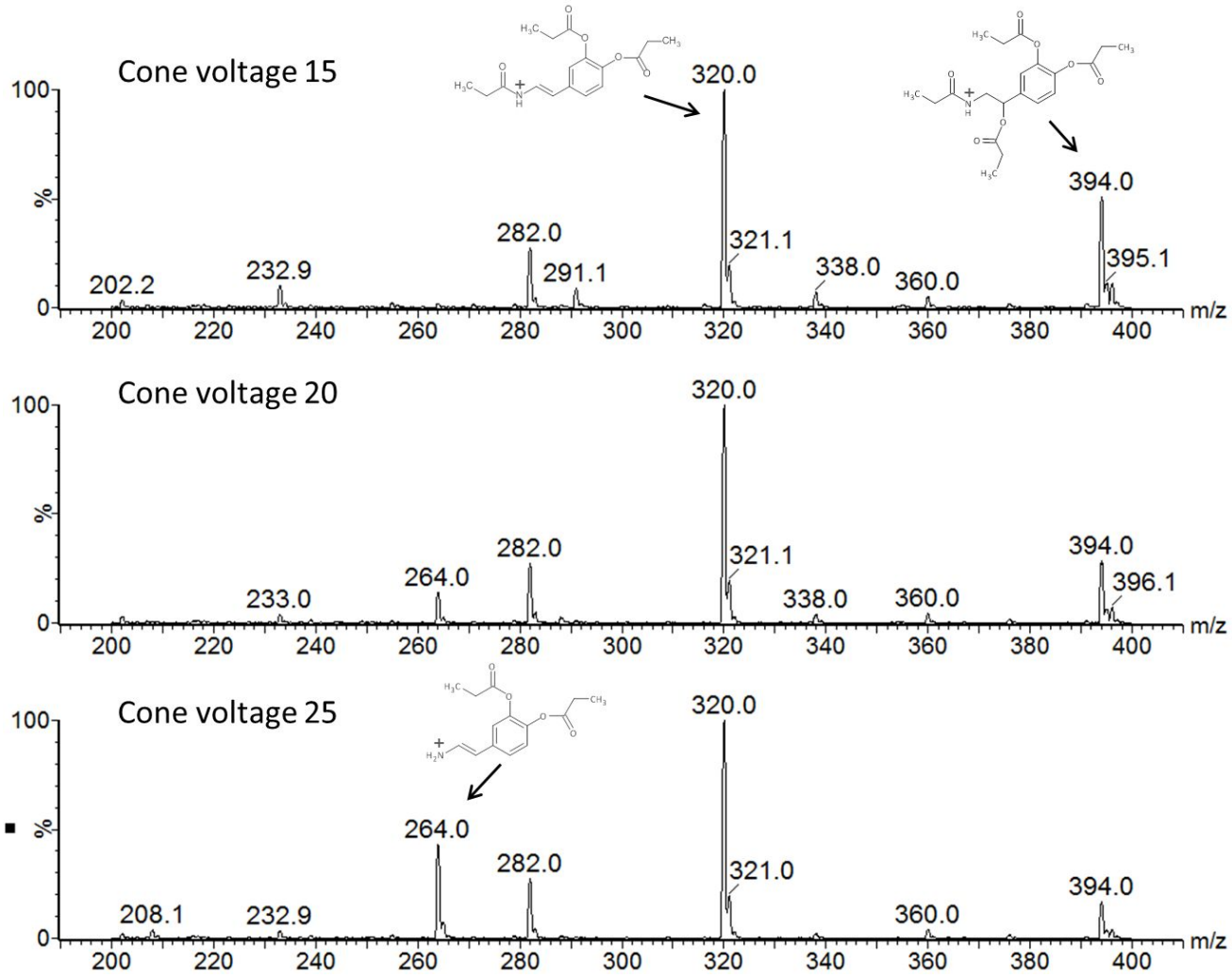


Figure S-5. Precursor spectrum of norepinephrine at different cone voltages.

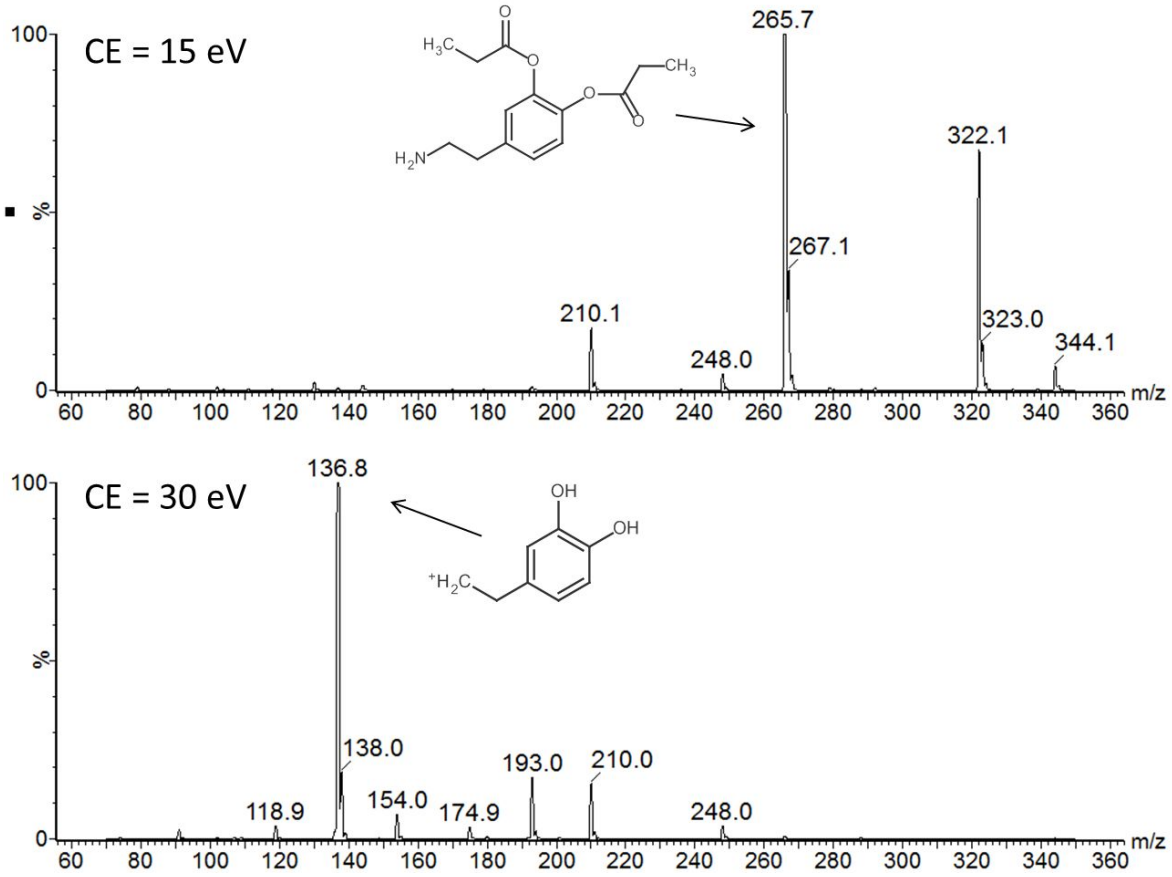


Figure S-6. Product ion spectrum of dopamine, m/z 322 at a collision energy of 15 eV and 30 eV. The proposed fragment for the most intense product is shown in the spectrum. Spectrum is normalized to the most abundant peaks. Y-axis denotes relative intensity (%), where the x-axis denotes the m/z values.

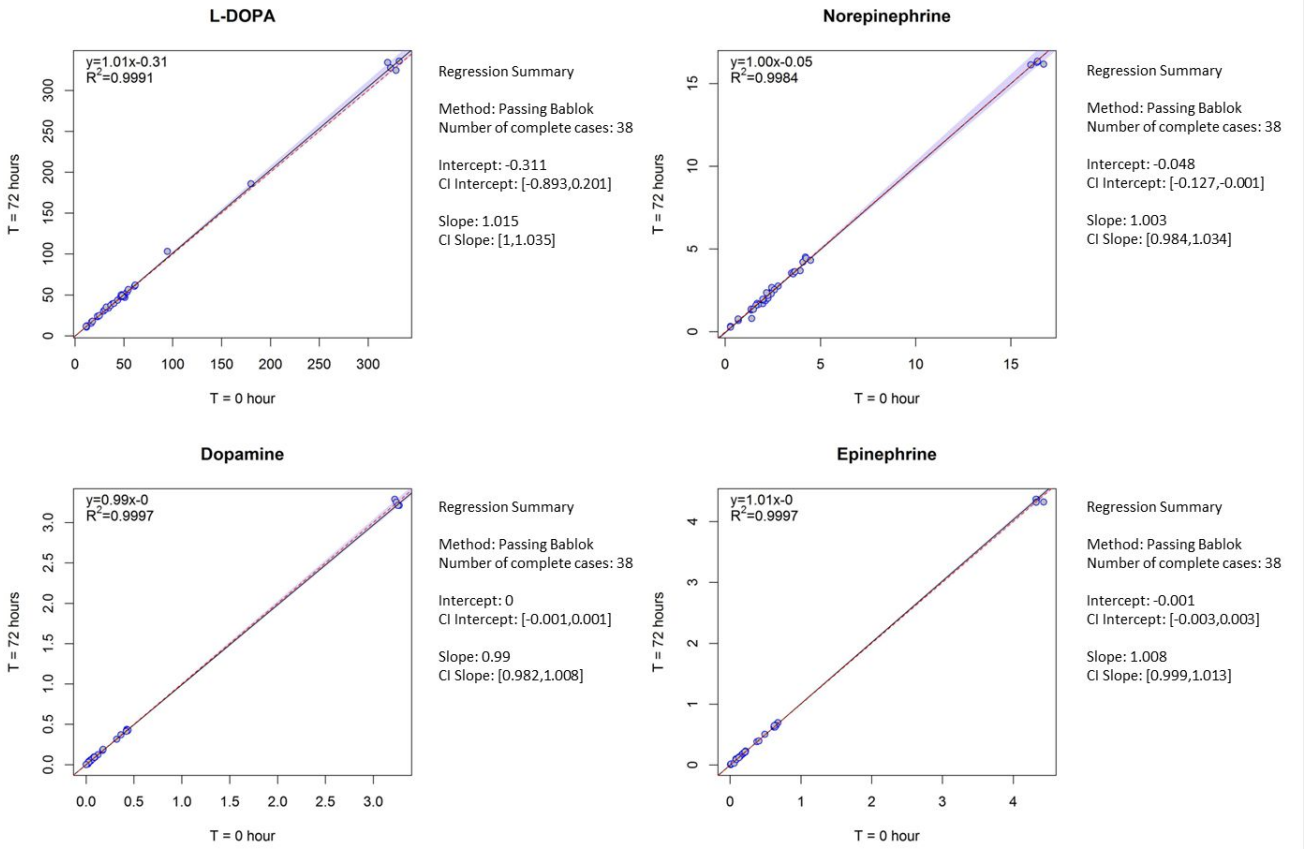


Figure S-7. Scatter plot of the autosampler stability results of catecholamines in 38 plasma samples. On the x-axis the concentrations of the respective catecholamine in nmol/L measured at T= 0 hour and on the y-axis the concentrations of the respective catecholamine in nmol/L measured at T = 72 hours by LC-MS/MS. Dashed red line represents the line of identity. Passing-Bablok regression analysis is shown by the solid line. Blue shaded area represents the 95% confidence interval. Abbreviations: LC-MS/MS, liquid chromatography tandem mass spectrometry; CI, 95% confidence interval.

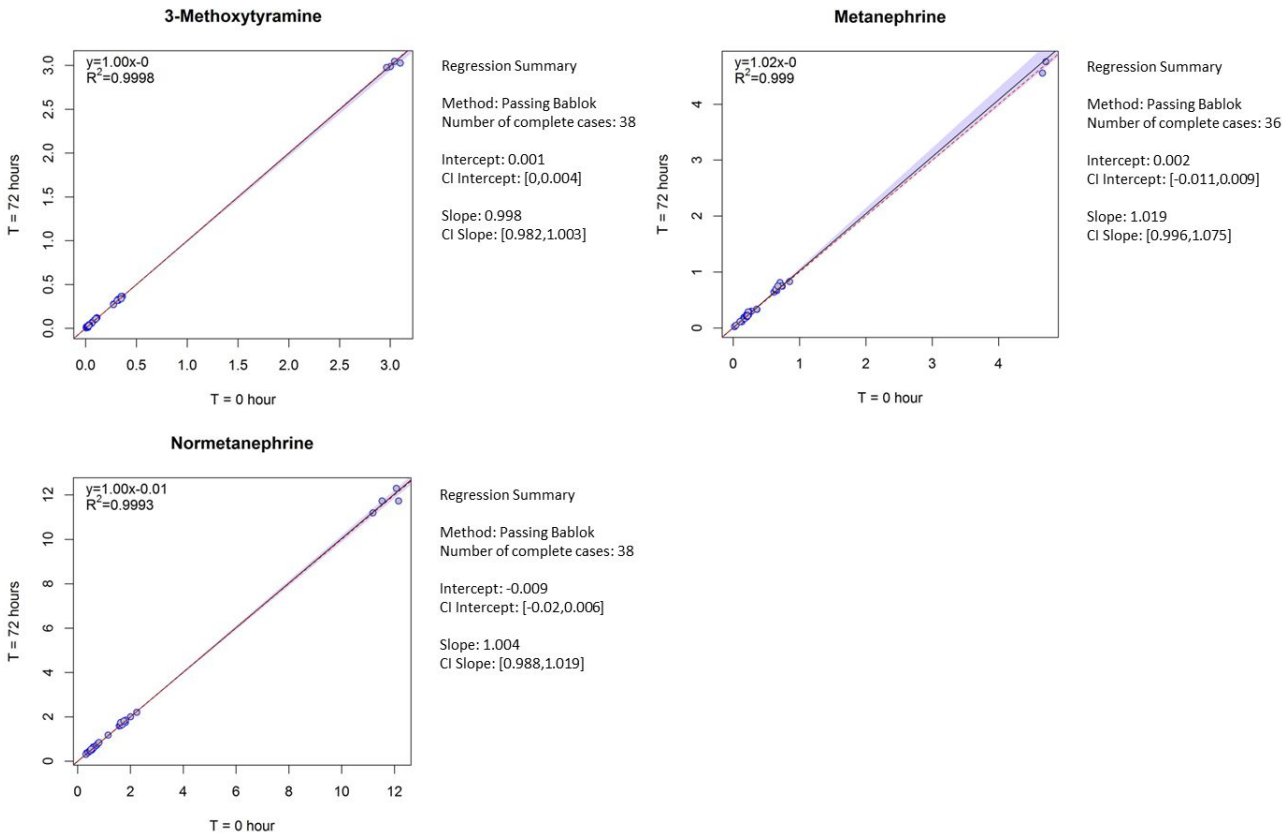


Figure S-8. Scatter plot of the autosampler stability results of metanephrines in 38 plasma samples. On the x-axis the concentrations of the respective metanephrine in nmol/L measured at T= 0 hour and on the y-axis the concentrations of the respective metanephrine in nmol/L measured at T = 72 hours by LC-MS/MS. Dashed red line represents the line of identity. Passing-Bablok regression analysis is shown by the solid line. Blue shaded area represents the 95% confidence interval. Abbreviations: LC-MS/MS, liquid chromatography tandem mass spectrometry; CI, 95% confidence interval.

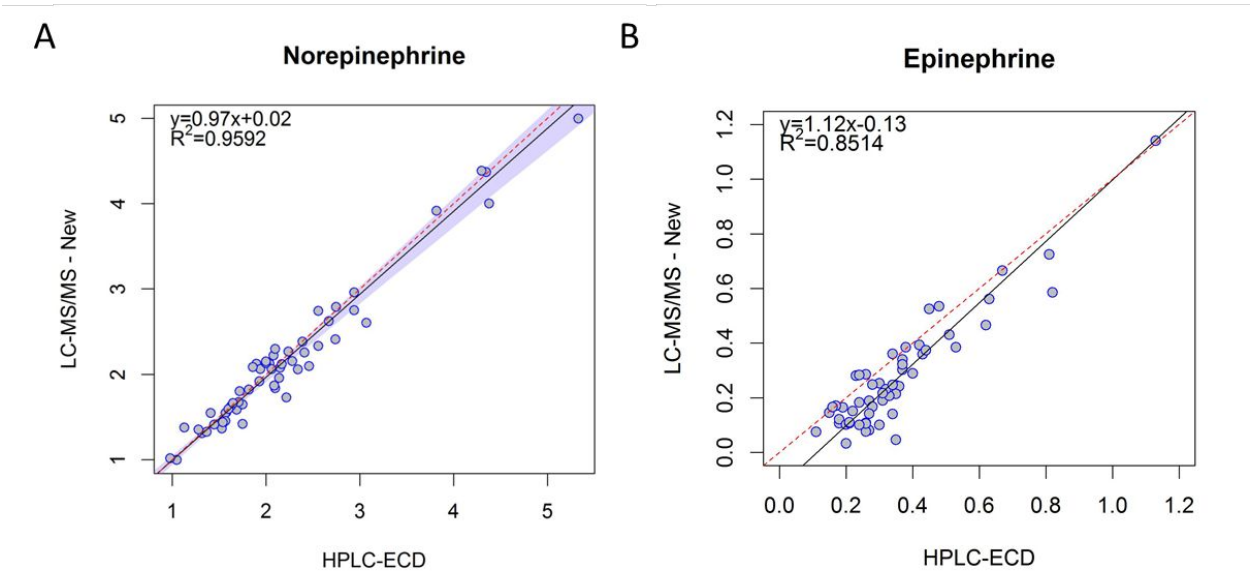


Figure S-9. Scatter plot for the method comparison of NE (Panel A) and E (Panel B) in 40 plasma samples. On the x-axis concentrations for NE and E measured by HPLC-ECD (in nmol/L) are shown and on the y-axis concentrations measured by LC-MS/MS (in nmol/L). Passing-Bablok regression gave a slope of 0.97 (95% CI 0.90–1.04), intercept of 0.022 (95% CI -0.105–0.146) for NE, and 1.12 (95% CI 0.97–1.29), intercept -0.13 (95%CI -0.188– -0.067) for E. Red dashed line represents the line of identity (x = y). Passing-Bablok regression analysis is shown by the solid line. Blue shaded area represents the 95% confidence interval. Abbreviations: E, epinephrine; NE, norepinephrine; HPLC, high-performance liquid chromatography; ECD, electrochemical detection.

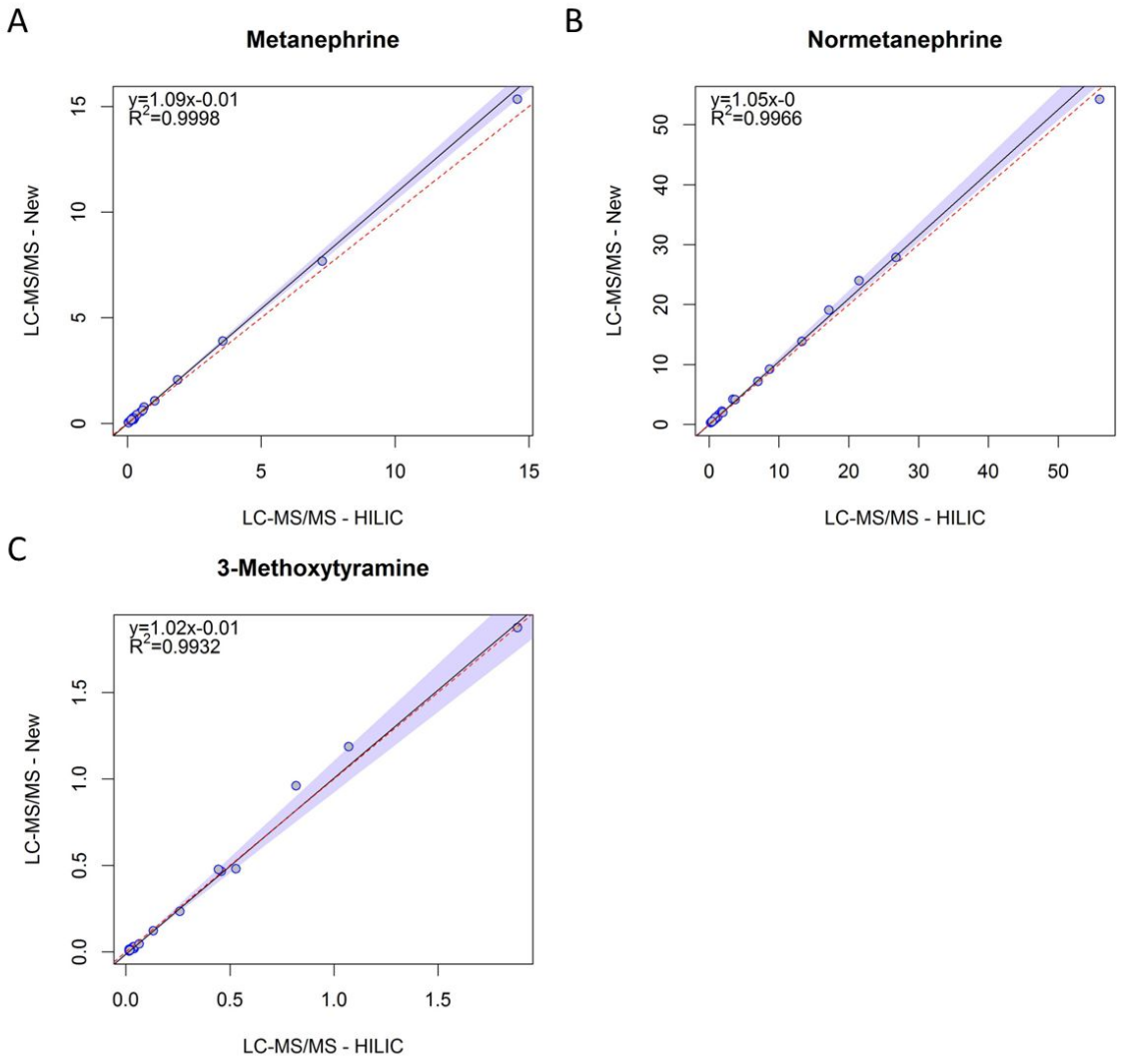


Figure S-10. Scatter plot for the method comparison of MN (Panel A), NMN (Panel B), and 3-MT (Panel C) in 40 plasma samples (n = 19 for 3-MT). On the x-axis concentrations for MN, NMN, and 3-MT measured by the HILIC LC-MS/MS method 15 (in nmol/L) are shown and on the y-axis concentrations measured by LC-MS/MS (in nmol/L) (current method). Red dashed line represents the line of identity. Passing-Bablok regression analysis is shown by the solid line. Blue shaded area represents the 95% confidence interval. Abbreviations: MN, metanephrin metanephrine, NMN, normetanephrine, 3-MT, 3-methoxytyramine, LC-MS/MS, liquid chromatography tandem mass spectrometry; HILIC, hydrophilic interaction liquid chromatography.

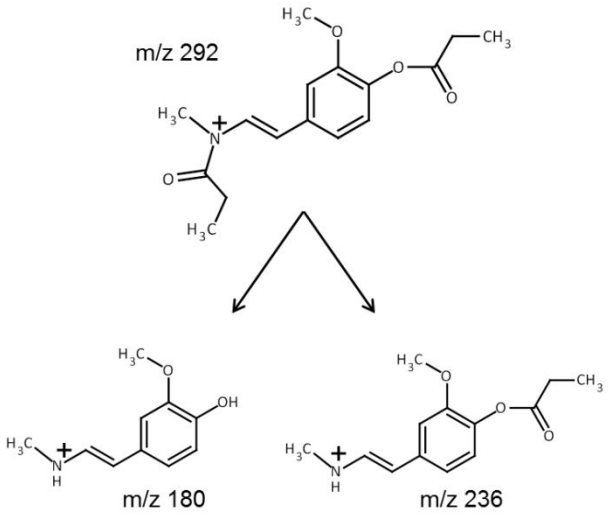


Figure S-11. Proposed fragmentation scheme for metanephrine.

DIALYZED PLASMA

Plasma from routine patient care was pooled and . dialyzed (Spectra/Por 3 Dialysis Membrane, MWCO 3.5 kDa, Fisher Scientific, Eindhoven, The Netherlands) against phosphate-buffered saline until no catecholamines or metanephrines were detectable. After dialysis, glutathione (reduced) was added to the plasma (~5 mg/mL). The dialyzed plasma was aliquoted and stored at -20 °C until use.

ONLINE SPE AND LC-MS/MS

Oasis HLB 10x1mm, 30 µm (Waters) cartridges were used for the online SPE procedure (see Figure S-7). Each cartridge was initially conditioned in the left clamp position with 500 µL acetonitrile, 500 µL of a mixture methanol/isopropanol/acetonitrile/water (1:1:1:1) containing 0.2% formic acid and then equilibrated with 500 µL water, at flow-rates of 5000 µL/min. Derivatized sample (100 µL) was aspirated and loaded onto the cartridge with 500 µL 0.1% formic acid at a flow-rate of 2000 µL/min. The two washing steps were performed with three different solvent compositions: wash 1) 500 µL 20% methanol, 4mM ammonium acetate and 0.4% formic acid, flow rate of 2500 µL/min; 2) 500 µL 20% methanol, 4mM ammonium acetate and 0.4% ammonia, flow rate of 2500 µL/min, and 3) 250 µL 20% acetonitrile, 4mM ammonium acetate and 0.4% formic acid, flow rate of 2500 µL/min. After washing, the cartridge was automatically transferred to the right clamp and the analytes were eluted by using the gradient elution option: The cartridge was eluted with the mobile phase starting gradient for 1:30 min. Following elution, the right clamp was flushed with 500 µL 40% acetonitrile in water, 0.2% formic acid at a flow rate of 5000 µL/min, 500 µL of a mixture methanol/isopropanol/acetonitrile/water (1:1:1:1) and 0.2% formic acid at a flow rate of 5000 µL/min, 500 µL acetonitrile at a flow rate of 5000 µL/min and finally 500 µL water at a flow rate of 5000 µL/min. A new cartridge was placed in the left clamp allowing the next sample to undergo SPE whilst chromatography was simultaneously being performed on the previous sample. The autosampler valve and needle were washed with 700 µL 10% acetonitrile in water, 750 µL 40% acetonitrile, 0.1% formic acid, followed by 750 µL mixture of methanol/isopropanol/acetonitrile/water, 4:2:2:2(v/v) and 0.2% formic acid and then 1000 µL 10% acetonitrile again.

METHOD VALIDATION

We evaluated intraassay imprecision by running twenty replicates of low, medium and high QC samples in a single run. Interassay imprecision was evaluated by analyzing replicates of low, medium and high level samples in 20 separate runs over a 3-month period. Recovery was determined by spiking low and medium QC samples with three different concentrations of the analytes on 6 different days. Recovery was calculated as follows: [(final concentration – initial concentration) / added concentration] * 100%. Lower limit of quantitation (LLOQ) for each analyte was determined by serial dilution of a low sample with dialyzed plasma (no endogenous analytes present) and analyzing the dilutions on six different days in duplicate. LLOQ was set where the precision was ≤ 20% and the signal to noise ratio > 10 [16]. Method validation was performed by evaluating imprecision, limit of quantitation, linearity, carryover, recovery, ion suppression, stability, and method comparison, which are described below. Quality control samples were prepared from left-over patient samples submitted for serotonin in PRP testing to our laboratory containing low, medium and high levels of the respective analytes. Quality control samples were stabilized with glutathione (~5 mg/mL) and stored at -80˚C until analysis. Concentrations of L-DOPA, dopamine, norepinephrine, epinephrine, 3-methoxytyramine, normetanephrine, and metanephrine in the quality control samples were 10, 47, 329 nmol/L; 0.095, 0.451, 3.33 nmol/L; 2.26, 4.48, 17. nmol/L; 0.216, 0.652, 4.37 nmol/L; 0.110, 0.326, 3.10 nmol/L; 0.534, 1.68, 12.1 nmol/L; 0.220, 0.667, 4.80 nmol/L, respectively. Carry-over was performed with the low and high quality control samples according to protocol EP10 from the Clinical and Laboratory Standards Institute [40]. Recovery was estimated by spiking catecholamines and metanephrines at three different levels to the quality control samples. These samples were analyzed on six different days. Plasma samples (n = 6) containing a low concentration of the analytes were analyzed as described above with constant post-column infusion of derivatives of the catecholamines and metanephrines at a flow-rate of 10 µL/min. Chromatograms of the samples were compared with those of the solvent blank and inspected for signs of ion suppression.

REFERENCE INTERVAL STUDY

A reference interval study was performed by analyzing 115 plasma samples from apparently healthy donors who gave informed consent. Inclusion criteria were: Subjects should be normotensive, defined as a blood pressure < 140/90 mmHg, without the use of antihypertensive medication. No documented cardiovascular history including: hypertension, diabetes, coronary artery disease, peripheral vascular disease. Exclusion criteria were: medication known to influence plasma catecholamines and metanephrines concentration: tricyclic antidepressants, phenoxybenzamine, MAO-inhibitors, sympathomimetics, cocaine, methyldopa). Blood samples were collected in the non-fasting state. Subjects were not allowed to smoke or drink caffeine containing beverages at least 12 hours in advance. After 30 minutes in supine position a blood sample was collected via direct venipuncture. All samples were collected using a Becton Dickinson Vacutainer® system with 10 mL EDTA coated tubes (both 1 x 10 ml BD Vacutainer EDTA K2E). Blood samples arrived at the laboratory within 60 minutes after withdrawal and were immediately centrifuged at 2,500 x g for 11 minutes after which the plasma was transferred into a cryovial (Sarstedt®) with glutathione and stored at -80 °C until analysis.