AUTOMATED DISPERSIVE PIPETTE EXTRACTION OF CATECHOLAMINES AND METABOLITES IN URINE USING THE HAMILTON MICROLAB® NIMBUS96® AND LC-MS/MS

HAMILTON®

CATECHOLAMINES

APPLICATION NOTE

The catecholamines, epinephrine, norepinephrine, and dopamine, are bioamines that play an integral role as neurotransmitters in the central and peripheral nervous system. Screening for catecholamines and their O-methylated metabolites, metanephrine and normetanephrine, is a widely accepted approach for diagnosis of catecholamine-secreting tumors, such as pheochromocytomas, neuroblastomas, and paragangliomas. Catecholamines are characterized by a monoamine-linked benzene ring with two vicinyl hydroxyl groups (catechol). Epinephrine is a secondary amine, while norepinephrine and dopamine are primary amines. Under neutral and alkaline conditions, the catechol group makes the catecholamines vulnerable to oxidation to the quinone species. Metanephrines lack a catechol group, having a methoxy group adjacent to the hydroxyl group, and are thus more stable. These compounds are highly polar and hydrophilic, with negative log D and log P values. These structural properties make sample preparation and analysis difficult.

We propose a diphenylborinic acid (DPBA) complexation with styrene divinyl benzene prior to dispersive pipette extraction (DPX) in order to minimize oxidation and maximize analyte recoveries from urine. Following elution, the complexation is reversed and the solution is ready for LC-MS/MS analysis. Automation of the DPX extraction using the Hamilton NIMBUS96® facilitates higher throughput by extraction of 96 samples in less than 15 minutes. The method described is highly reproducible and provides the necessary sensitivity for clinical applications.

WORKFLOW

A well plate was prepared with 300 µL of sample, spiked with internal standard (10 µL). The well plate was loaded onto the Hamilton NIMBUS96®. Reservoirs of diphenylborinic acid (0.2% (w/v), 5 g/L EDTA in a 2 M NH_4Cl/NH_4OH pH 8.5 buffer) as complexing agent, wash buffer (0.2 M NH_4Cl/NH_4OH pH 8.5), 100% methanol, and 1 M formic acid (FA) were also added to the deck of the automated platform. The liquid handling system used CO-RE 1 mL tips to add 600 µL of complexing agent to the urine sample well plate, 500 µL of wash buffer to a second "wash" well plate, 270 µL of 1 M formic acid and 30 µL of methanol to the third "elution" well plate. The Hamilton robotic system discarded the CO-RE tips and picked up 1 mL DPX reverse phase (RP) tips. After conditioning by twice aspirating and dispensing 100% methanol, the tips were conditioned with wash buffer, and the sample solution was aspirated and dispensed four times. The elution well plate was then moved to the autosampler for LC-MS/MS injection.

1 COMPLEX ANALYTES	*Add DPBA to Urine*
2 CONDITION	*100% MeOH* *0.2 M NH_4Cl pH 8.5*
3 BIND ANALYTES	*Aspirate/Dispense Sample*
4 WASH	*0.2 M NH_4Cl pH 8.5*
5 ELUTE ANALYTES	*10% MeOH in 1 M FA*

Note that the DPBA complexation is essentially quantitative. Repeated aspiration and dispensing of the sample solution insures complexation and efficient retention on the styrene divinyl benzene resin. After rinsing the tips with the wash buffer, analytes were eluted with the 1 M formic acid/10% methanol solution. Acidification reverses the diphenyl boronate complexes, while methanol enhances elution by disrupting any reverse phase interactions. Low methanol content is also beneficial for minimal removal of any retained matrix thus maximizing selectivity.

Analyses were performed using a Thermo TSQ Vantage™ triple quadrupole mass spectrometer coupled to an Agilent 1260 Series HPLC equipped with a Restek 3 µm Ultra PFPP column (100 mm × 2.1 mm) with column temperature held at 40 °C and a 10 µL injection volume. The mobile phase was composed of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). The most abundant *m/z* was monitored for each compound with the M+H ion for epinephrine (183.9) and dopamine (153.8) and the water-loss product ions ($M+H-H_2O$) for norepinephrine (151.8),

normetanephrine (165.8), and metanephrine (180.0).

RESULTS AND DISCUSSION

Accuracy, within-run, and between-run precision (% CV) are reported. Method validation was performed using ClinChek® lyophilized biogenic amine controls (RECIPE, Munich, Germany). The method was very accurate for quantitation of quality control samples with each average analyte concentration falling within the manufacturer's expected range of concentrations. Average within-run precision demonstrated a maximum CV of 6% for the Level 1 epinephrine control, while between-run precision had a maximum CV of 7% for the Level 2 metanephrine control (Table 1).

Calibrations resulted in average coefficients of determination (R^2) values of 0.9992 for norepinephrine, 0.9996 for epinephrine, 0.9996 for dopamine, 0.9998 for normetanephrine, and 0.9982 for metanephrine. The limit of detection (LOD) was calculated using the estimated standard deviation of the y-intercept (S_y) and the average slope of the calibration (Avg_m): LOD=(3.3 S_y)/Avg_m. The limit of quantitation (LOQ) was calculated using: (10 S_y)/Avg_m. Results are shown in Table 2.

Matrix effects were relatively low for epinephrine, dopamine, normetanephrine, and metanephrine with a range of ion suppression from 1–14%. Norepinephrine exhibited greater matrix effects with ion suppression at 39%, likely because it is the most polar compound with little to no retention on the PFPP column. Extraction efficiencies were greater than 96% for all analytes except dopamine, which demonstrated an 81% recovery.

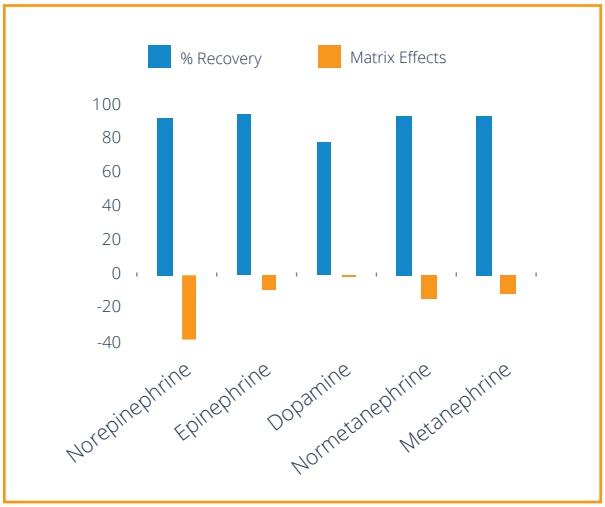

Figure 1. Recovery and matrix effects of catecholamines and metanephrines extracted using automated DPX method.

Table 1. Accuracy and precision of the method based on two levels of external quality control.

Analyte	Expected Range	Mean	Within-Run, CV	Between-Run, CV
Level 1				
Norepinephrine	51.2–76.8	62.8	5%	5%
Epinephrine	16.0–24.0	19.6	6%	5%
Dopamine	155–233	186.2	3%	6%
Normetanephrine	260–390	264.5	5%	5%
Metanephrine	138–206	166.6	5%	7%
Level 2				
Norepinephrine	139–209	168.3	3%	5%
Epinephrine	31.8–47.8	39.7	2%	2%
Dopamine	234–352	284.3	4%	5%
Normetanephrine	1254–1882	1493.7	4%	4%
Metanephrine	814–1220	1056.8	3%	4%

Table 2. The standard deviation of the y-intercept (S_y), average slope (Avg_m, n=5), limit of detection (LOD) in ng/mL, limit of quantitation (LOQ) in ng/mL, and the average coefficient of determination (R^2, n=5).

Compound	S_y	Avg_m	LOD	LOQ	Avg R^2
Norepinephrine	0.0037	0.068	0.18	0.53	0.9992
Epinephrine	0.0058	0.088	0.22	0.65	0.9996
Dopamine	0.0055	0.12	0.15	0.46	0.9996
Normetanephrine	0.00019	0.19	0.003	0.01	0.9998
Metanephrine	0.0016	0.17	0.03	0.09	0.9982

CONCLUSION

We report a rapid and robust analytical method that achieves sensitive LC-MS/MS analysis of free catecholamines and metanephrines in urine. Dispersive pipette extraction facilitates seamless integration of SPE with the Hamilton Microlab® NIMBUS96® for extraction of DPBA complexed catecholamines and metanephrines from urine in less than 15 minutes. This method provides the necessary analytical sensitivities without an additional solvent evaporation step. The calibration was linear (R^2 > 0.998) over more than four orders of magnitude with concentrations ranging from 0.5–1000 ng/mL. Replicate analyses of two different levels of synthetic urine controls demonstrated % CV of less than 8%. The method is an excellent alternative to those previously published, given ease of implementation, robustness, high sensitivity due to enhanced sample clean-up, and high throughput with a LC-MS/MS run time of 5.5 minutes.