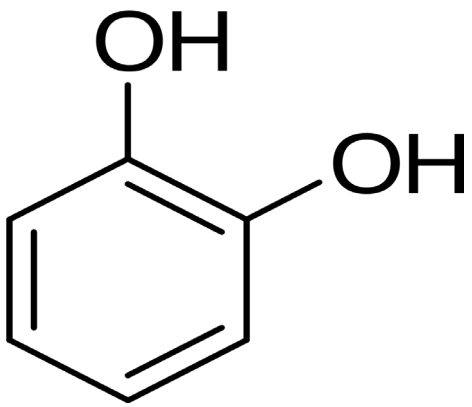
Simultaneous Quantitative Analysis of Total Catecholamines and Metanephrines in Urine Using 500 MG CLEAN-UP® CCX2 and LC-MS/MS



UCT Part Numbers

CUCCX256
Clean-Up CCX2
(C8 + Carboxylic Acid)
500mg/6mL

SLGRDHLDR-HPOPT
Guard cartridge holder

SLPFPP100ID21-3UM
Selectra® PFPP HPLC column
100 x 2.1mm, 3um

SLPFPPGDC20-3UM
Selectra® PFPP guard column
10 x 2.0mm, 3um

Introduction:

Catecholamines are hormones produced by the cells in the interior region of the adrenal glands. These hormones function in the control of heart rate, metabolism and blood pressure (1). The primary catecholamines are dopamine, norepinephrine, and epinephrine. Metanephrine and normetanephrine are the 3-methoxy metabolites of epinephrine and norepinephrine, respectively. Elevated catecholamine levels are commonly associated with stress; however in patients with pheochromocytoma or paragangliomas, these hormones are released into the body without being triggered by any outside factors (2).

Pheochromocytoma is a condition in which a neuro-endocrine tumor develops in the medulla of the adrenal glands. It originates from the chromaffin cells and results in uncontrolled secretion of epinephrine and other catecholamines. The classic symptoms of pheochromocytomas are those attributable to excess adrenaline production. Patients often experience episodes of sweating, headache, hypertension and heart palpitations. Pheochromocytomas are rare and usually benign; however, in about 10% of cases, these tumors can be malignant (2). When a tumor of chromaffin cells occurs outside of the adrenal glands it is known as a paraganglioma. These tumors produce the same effects as pheochromocytomas and can form anywhere in the body.

Patients who are thought to have a pheochromocytoma are often diagnosed following biochemical tests that measure the levels of catecholamines and metanephrines. Metanephrines are stable metabolites of catecholamines that are co-secreted directly with catecholamines from these tumors. While catecholamines only last a short time in the body before they are metabolized, metanephrines last much longer making them a more informative marker to indicate an abnormality (3). Catecholamine and metanephrine levels may be measured in either plasma or in urine that has been collected over a 24 hour period. Generally, plasma tests are preferred due to the enhanced sensitivity of this assay, but there is elevated risk of reporting false positives utilizing this matrix. The 24 hour urine test is considered to be much more conclusive. The criterion used to deem a sample positive for pheochromocytoma is metanephrine values twice the average upper limit (1).

By utilizing UCT's CLEAN UP® CCX extraction columns along with a Selectra® PFPP HPLC column, excellent sample clean up and analyte separation w as achieved. This was demonstrated by the observation of good recovery for all five of the hormones analyzed.

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Procedure:

1. Prepare Sample

a) To 1 mL of urine add internal standard(s) and 100 µL of 6M HCl.
b) Incubate for 20 minutes at 90 °C for 20 minutes.
c) Neutralize pH with NaOH (~ 75 µL) and 3mL of Acetate buffer (pH ~ 7.0).
d) Mix/Vortex.

2. Condition Column

a) 1 x 3 mL MeOH
b) 1 x 3 mL DI H_2O
c) 1 x 3 mL Acetate Buffer (pH 7)

3. Apply Sample

a) Load at 1 to 2 mL/minute.

4. Wash Column

a) 1 x 3 mL DI H_2O
b) 1 x 3 mL 50:50 MeOH:ACN
c) Dry column under full vacuum or pressure for 10-15 minutes.

5. Elute Analytes

a) 1 x 3 mL MeOH w / 5% formic acid
b) Collect eluate at 1-2 mL/ minute.

Note: A 1% HCl in MeOH solution has been used to prevent volatilization by the formation of the hydrochloric salt of the drugs.

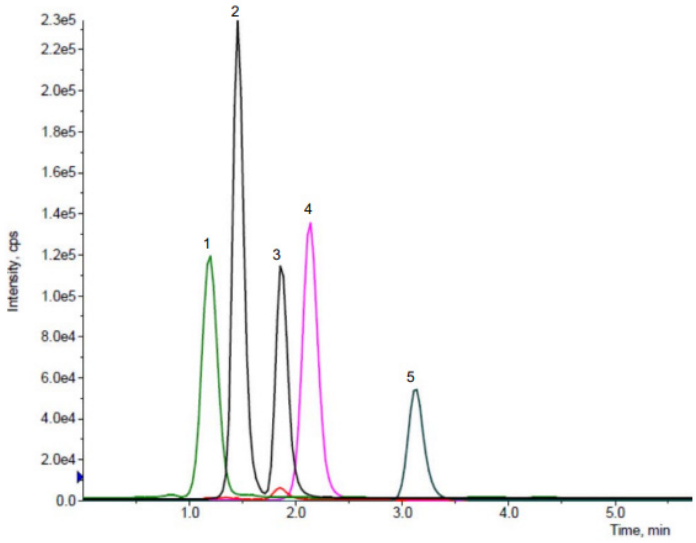
6. Dry Eluate

a) Evaporate to dryness at < 40 °C.

7. Reconstitute

a) Reconstitute sample in 100 µL of mobile phase.

Chromatogram -- Catecholamine Standard – 100ng/mL



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MRM Transitions				
Analyte		Retention Time (min)	Q1	Q3
1	Norepinephrine	1.19	170.4	152.0
2	Epinephrine	1.45	184.0	166.0
3	Normetanephrine	1.85	166.9	135.0
4	Dopamine	2.12	153.9	136.9
5	Metanephrine	3.12	180.0	165.0

LC-MS/MS Method	
Instrument	Agilent 1200 Binary Pump SL
Detector	AB Sciex API 4000 QTrap MS/MS
Column	UCT Selectra® PFPP column, 100 x 2.1 mm, 3 µm
Guard Column	UCT Selectra® PFPP, 10 x 2.0 mm, 3 µm
Column Temperature	50 °C
Flow Rate	0.3 mL/min
Injection Volume	10 µL

Gradient Program		
Time (min)	% Mobile Phase A (0.1% FA in water)	% Mobile Phase B (0.1% FA in Methanol)
0	99	1
2	99	1
3	0	100
5	0	100
5.2	99	1
10	99	1

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Results:

Recovery (n=3)			
Analyte	**75 ng/mL**	**500 ng/mL**	**2000 ng/mL**
Epinephrine	86%	88%	84%
Norepinephrine	101%	91%	79%
Metanephrine	79%	78%	63%
Normetanephrine	49%	42%	34%
Dopamine	97%	91%	86%

Discussion:

The structures and pKa values of epinephrine, norepinephrine, metanephrine, normetanephrine and dopamine make them ideal candidates for clean-up via cation exchange solid phase extraction (SPE). Two chemically different SPE columns with various combinations of wash and elution solvents were evaluated for the optimization of this procedure. UCT's benzenesulfonic acid strong cation exchange column and corresponding methodology allow ed for excellent recovery of the metanephrines, how ever, it greatly reduced the recovery of the catecholamines regardless of the wash/elution solvent combination. Previous literature suggests that the use of highly basic elution solvents containing ammonium hydroxide lead to degradation of the catecholamines, which was noted in the extraction results. In an attempt to improve recovery, a 2% trimethylamine / 98% methanol solution was substituted for elution purposes and no improvement was noted.

Subsequently, UCT's CLEAN UP® CCX was selected. This is a carboxylic acid, weak cation exchange, sorbent. It allowed for adequate recovery of the metanephrines w ithout compromising the stability of the catecholamines. Samples were loaded at pH 7, ensuring that the functional groups of both the analytes of interest and the sorbent were fully charged. The charged state allowed for strong ionic interactions between the sorbent and the catecholamines. The ionic interaction permitted the use of an extensive range of wash solutions. Ultimately, it was determined that a D.I. H_2O wash follow ed by an acetonitrile: methanol (50:50) wash best removed unwanted matrix and other endogenous interferences.

For elution purposes, an acidic solution (5% formic acid in methanol) sufficiently lowered the pH of the sorbent. This neutralized the carboxylic acid groups on the sorbent and disrupted the ionic bonds with the analytes of interest. The analytes were now able to be efficiently removed from the SPE column.

Prior to evaporating to dryness, 100 µL of a 1% HCl in methanol solution was added to the diluent to prevent volatilization of the analytes.

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Conclusions:

1. Both catecholamine and metanephrine levels need to be monitored. This is done for both the diagnosis of a patient with pheochromocytoma, and also to monitor treatment for patients who are already known to have the condition.
2. By utilizing UCT's CLEAN UP® CCX extraction columns and corresponding methodology, total urine catecholamine and metanephrine levels can be monitored simultaneously, reducing both analyst time and instrument time.
3. UCT's PFPP analytical column allow ed for the simultaneous separation of the extremely polar catecholamines and metanephrines. Of the various HPLC phases tried (pentafluorophenylpropyl, polyaromatic, aqueous C18, and Diol) and conditions attempted, the Selectra® PFPP (pentafluorophenylpropyl) column was the only one that produced acceptable baseline separation and peak shape for the quantitation of all analytes.
4. It is strongly recommended to use matrix-matched calibration curves, which include isotopically labeled internal standards to compensate for any remaining matrix that is not removed via the extraction procedure.

References:

[1] "Catecholamines in Urine." WebMD. WebMD, 20 June 2012. Web. 15 June 2015.

[2] "Pheochromocytoma: MedlinePlus Medical Encyclopedia." U.S National Library of Medicine. U.S. National Library of Medicine, 15 June 2015. Web. 16 June 2015.

[3] "Pheochromocytoma." - Mayo Clinic. N.p., 02 May 2014. Web. 22 June 2015.

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