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A Simple, Effective Method for Quantitative Analysis of the Biomarker Norepinephrine in Human Plasma by LC-MS/MS

Bao Hoang, Casey Bonner, Alicia Pietrasiewicz, and Steven Wiltshire
Agilux Laboratories – A Charles River Company, Worcester, MA

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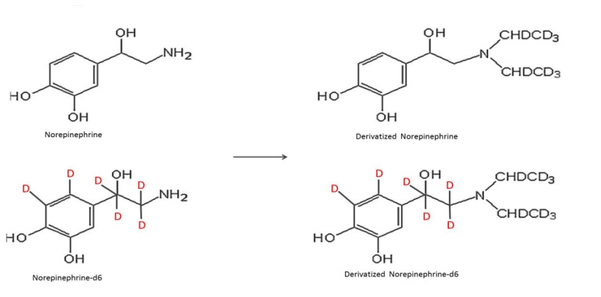
PURPOSE

Measuring endogenous catecholamines such as norepinephrine in plasma is important in the discovery, evaluation, and monitoring of drugs in many disease/disorder areas including diabetes, cancer (for example neuroblastoma), heart disease, pain, and anxiety. Quantification of catecholamine concentrations in plasma can provide a direct biomarker measurement of disease state and/or proper target engagement. Norepinephrine analysis in plasma has challenges including but not limited to; low concentrations, instability, and interferences. We were able to address the challenges and qualify a simple, fast, and effective method for quantitative analysis of norepinephrine in human plasma.



Derivatized Compound	Q1 m/z	Q3 m/z
Norepinephrine	234.1	183.0
Norepinephrine-d6	240.1	189.1

METHOD

Table 1. LC Method for Norepinephrine

Time (min)	%MPA	%MPB
0.20	95	5
1.10	75	25
1.11	5	95
2.40	5	95
2.41	95	5
3.00	95	5
MPA: 20 mM Ammonium Acetate in 100:0.2 (v:v) Water:Formic acid		
MPB: Methanol		

METHOD (Continued)

Norepinephrine is an endogenous compound in plasma. Given a variety of considerations including norepinephrine instability, we chose a "depleted matrix" approach for quantitation. This approach consists of four key features: (1) calibrants and QCs are prepared in plasma from which endogenous norepinephrine is removed as described below, (2) true analyte is utilized for spiking calibrants and stable-label norepinephrine is utilized as internal standard, (3) derivatization with d4-acetaldehyde for improved chromatography, sensitivity, and stability, and (4) additional stability considerations including sodium metabisulfite addition, with on-ice and yellow-light extraction conditions.

To generate the "depleted matrix" for calibrants and QCs, K2 EDTA human plasma was exposed to light while gently mixing for a minimum of 48 hours, after which the elimination of endogenous norepinephrine was confirmed. Extracts were prepared by conventional acetonitrile-based protein precipitation and were then derivatized with d4-acetaldehyde. Analysis was performed utilizing Agilent 1290 UHPLC pumps, a Leap CTC Pal autosampler, and Sciex API6500. Waters UPLC BEH C18 column (50 x 2.1 mm, 1.7 µm) was used for LC separations. Mobile phases consisted of 20 mM ammonium acetate in water: formic acid (mobile phase A) and methanol (mobile phase B). Gradient chromatography was employed for separation and total run-time was 3 minutes at a flow rate of 0.7 mL/min. Sciex Analyst 1.6 was utilized for integrations and calculations.

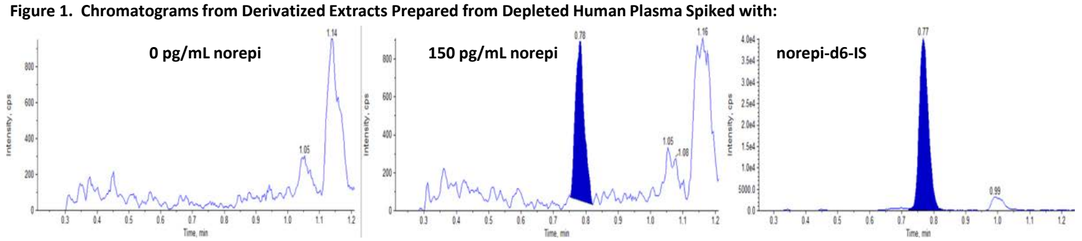
RESULTS

The method was successfully qualified using a combination of true analyte, depleted matrix, and stable-labeled internal standard. The qualified assay range is 0.0500 ng/mL to 100 ng/mL, and the assay met all parameters evaluated including intra-day accuracy/precision, inter-day accuracy/precision, carryover, dilution linearity, selectivity, and specificity. In addition, in-process stability was evaluated and addressed, including the time required for blood to plasma conversion as well as subsequent stability in plasma during handling and freeze/thaws. Representative qualification data (calibrants and batch acceptance QCs) are shown in the following two tables along with a representative norepinephrine calibration curve from Analyst®. The method has been utilized successfully for analysis of a variety of study samples.

Table 2. Summary of Derivatized Norepinephrine Inter-batch Statistics for Calibration Standards

Nominal Conc. (ng/mL)	STD1	STD2	STD3	STD4	STD5	STD6	STD7	STD8	STD9	STD10
	0.0500	0.100	0.250	0.500	2.50	5.00	10.0	25.0	50.0	100
Mean	0.0516	0.0940	0.250	0.492	2.51	5.02	9.98	25.2	51.1	101
SD	0.00577	0.00737	0.0137	0.0152	0.0481	0.141	0.117	0.423	0.993	2.96
%CV	11.2%	7.8%	5.5%	3.1%	1.9%	2.8%	1.2%	1.7%	1.9%	2.9%
% Bias	3.1%	-6.0%	0.0%	-1.7%	0.3%	0.4%	-0.2%	0.9%	2.1%	1.0%
n	8	8	8	8	8	8	8	8	8	8

Figure 1. Chromatograms from Derivatized Extracts Prepared from Depleted Human Plasma Spiked with:



RESULTS (Continued)

Figure 2. Representative Norepinephrine Calibration Curve

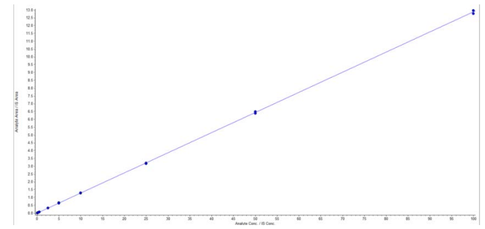
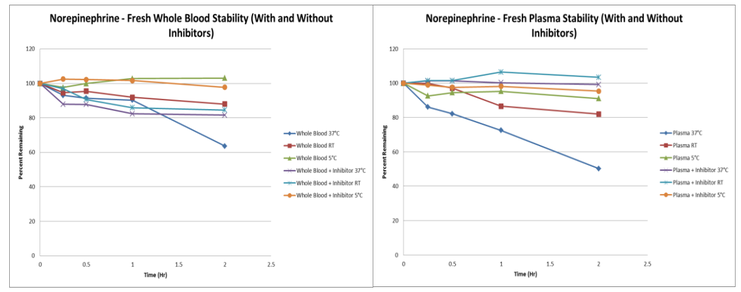


Table 3. Derivatized Norepinephrine QC Concentrations with Inter-batch Summary Statistics

Nom. Conc. (ng/mL)	QC-LLOQ	QC-Low	QC-Mid	QC-High	QC-Dil*
	0.0500	0.150	2.00	80.0	500
Mean	0.0532	0.166	2.17	84.6	521
SD	0.00325	0.0127	0.0752	2.15	3.77
% CV	6.1%	7.7%	3.5%	2.5%	0.7%
% Bias	6.4%	10.4%	8.7%	5.8%	4.2%
n	3	11	11	11	4
*20-fold dilution					

Figure 3. Anti-oxidant Plus 5°C Conditions Stabilizes Norepinephrine in Human Plasma



CONCLUSION

A simple, effective method was developed with a 50 pg/mL lower limit of quantification utilizing conventional protein precipitation coupled with derivatization with d4-acetaldehyde. This method enables measurement of norepinephrine biomarker levels in normal, disease state, and treated-patients in support of a variety of clinical studies.

REFERENCE

"Simultaneous Determination of Plasma Epinephrine and Norepinephrine using an Integrated Strategy of a Fully Automated Protein Precipitation Technique, Reductive Labeling and UPLC-MS/MS". Chengjie Ji, Justin Walton, Yi Su, Max Tella, Analytica Chimica Acta, 670 (2010) 84-91