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I Knew You Were Trouble: Trials and Tribulations of Method Development for Extended Pathway, Low-Volume, Plasma Catecholamine Assay by LC-MS/MS

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Introduction

Catecholamines are metabolized from tyrosine and play important roles in neuromodulation in the central nervous system and in moderating human behavior. Their levels fluctuate considerably over the course of the day. Because catecholamines play such a varied and important role in the healthy functioning of the body, there has been consistent interest in methods for their detection. However, they are of low abundance in plasma and are hydrophilic, which creates difficulty in developing analytical methods, especially those that use smaller volumes of samples. This work explores method development for a reduced volume procedure to detect 18 catecholamines, precursors, and metabolites in plasma.

Compounds included in this study were dopamine, epinephrine, norepinephrine, L-DOPA, 3-methyoxytyramine, metanephrine, normetanephrine, octopamine, synephrine, tyramine, 5-hydroxyindoleacetic acid, homovanillic acid, vanillylmandelic acid, phenylalanine, tyrosine, N-methyl-tyramine, N-methyl-phenethylamine, and phenethylamine (structures shown in Figure 1). There were four sets of isobars that had to be resolved, which was difficult to achieve with a standard reverse phase regime; however, an exploration of non-standard column chemistries and the addition of ion pairing reagents directly into the injection vials demonstrated promising results.

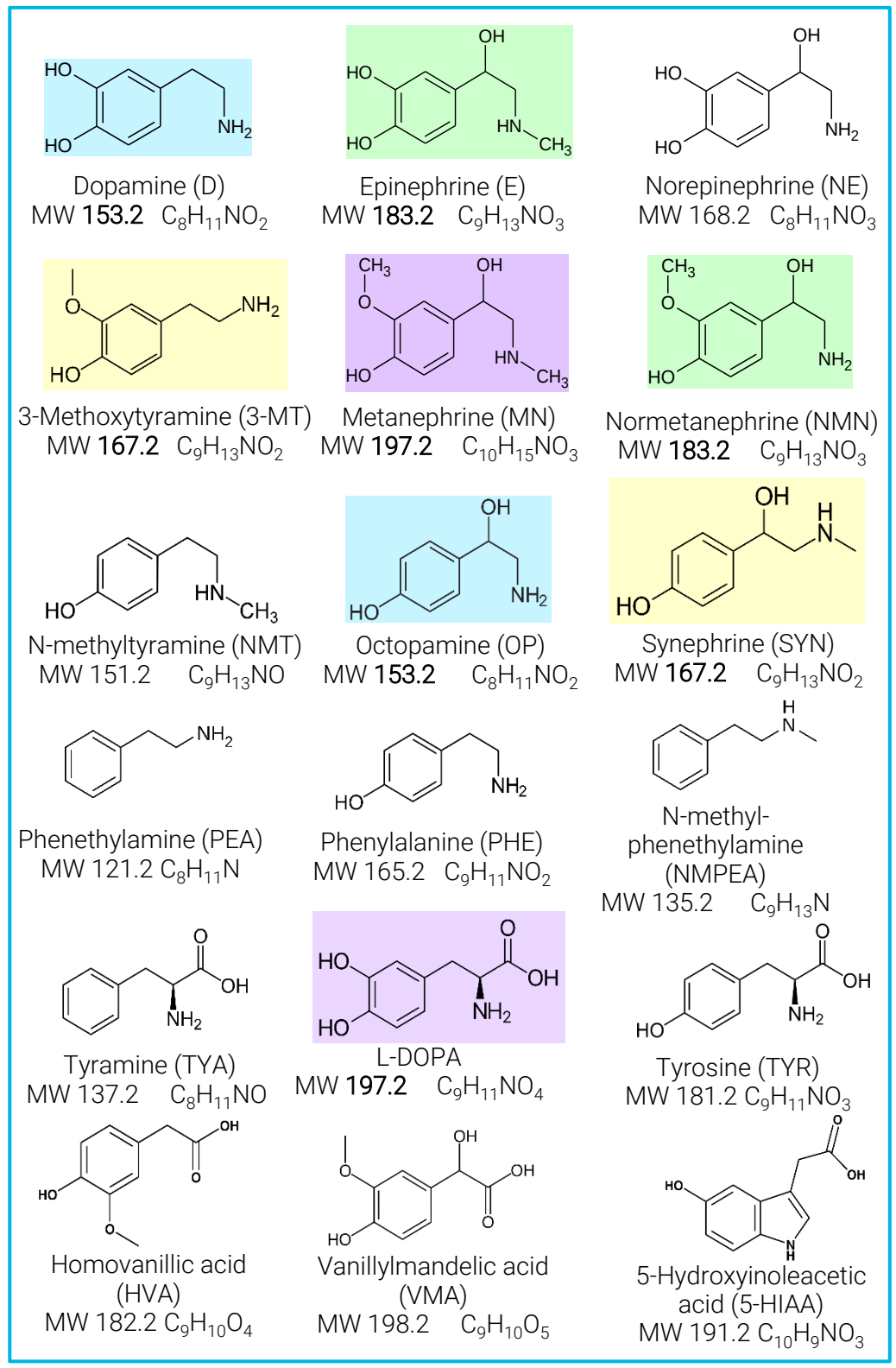

Figure 1. Structures of compounds in catecholamines biochemical pathway.

Experimental

Standards and Curve Preparation

Plasma was purchased from Golden West Biologicals, while analytes and internal standards were purchased from Cerilliant and Sigma-Aldrich. Plasma was spiked with a mix of 18 catecholamines, metabolites, and precursors from a working stock solution, and following sample cleanup, 5 µl was injected into the LC-MS/MS system.

LC-MS/MS Analytical Method

To verify sensitivity, the method was tested on two different mass spec models.

The LC-MS/MS systems consisted of a 1290 Infinity II UHPLC stack with a binary pump, a thermostatted multisampler, and a temperature-controlled column compartment. The detectors were a 6495C or an Ultivo mass spectrometer. Separation conditions are shown in Table 1. Detection of all analytes was undertaken in multiple reaction monitoring (MRM) mode. MS source conditions for each detector are shown in Table 2. The total injection cycle time was approximately 12 minutes. Data was acquired and analyzed using MassHunter software suite version B.10.1 for data collection from the 6495 or version 1.2 for data collection from the Ultivo.

Experimental

Column	Agilent Poroshell 120 EC-C18, 2.1 x 100 mm, 1.9 µm	
Injection Volume	5 µL	
Mobile Phase A	H_2O + 0.1% Formic Acid	
Mobile Phase B	Methanol	
Needle Wash	50:20:20:10 IPA:MeOH:ACN:H2O	
Autosampler Temp	5 °C	
Column Temp	55 °C	
Flow Rate	0.3 mL/min	
Gradient	0.00 min 2.00 min 4.00 min 6.00 min 7.00 min 7.01 min	0% B 0% B 10% B 98% B 98% B 0% B
Stop Time	9.5 min	
Post Time	1.5 min	

Table 1. UHPLC conditions.

	Ultivo (+/-)	6495 (+/-)	Units
Gas Temp	150	150	°C
Gas Flow	12	14	L/min
Nebulizer Pressure	35	35	psi
Sheath Gas Temp	350	350	°C
Sheath Gas Flow	11	11	L/min
Capillary Voltage	2000/3000	2000/3000	V
Nozzle Voltage	0/1500	0/1500	V
Funnel	N/A	Hi: 80/90 Lo: 130/60	V
Delta EMV	0	0	V

Table 2. AJS source conditions.

Agilent Ultivo and 6495 triple quadrupole LC/MS systems

Results and Discussion

Chromatography

Historical LC-MS/MS methods for the detection of catecholamines focused on three analytes: dopamine, epinephrine, and norepinephrine. These compounds are often analyzed at the low picogram level, and they don't retain well on typical reverse-phase columns. Interferences are also a concern with these analytes, as they are isobars of other precursors and metabolites found in the catecholamine pathway. With these issues in mind, work was undertaken to adjust the chromatography that had previously been worked out to see if retention could be improved for the three primary catecholamines, as well as to see if resolution between the isobaric pairs could be achieved for the additional compounds in the pathway. The original chromatography is shown in Figure 2, while example adjusted chromatography is shown in Figure 3.

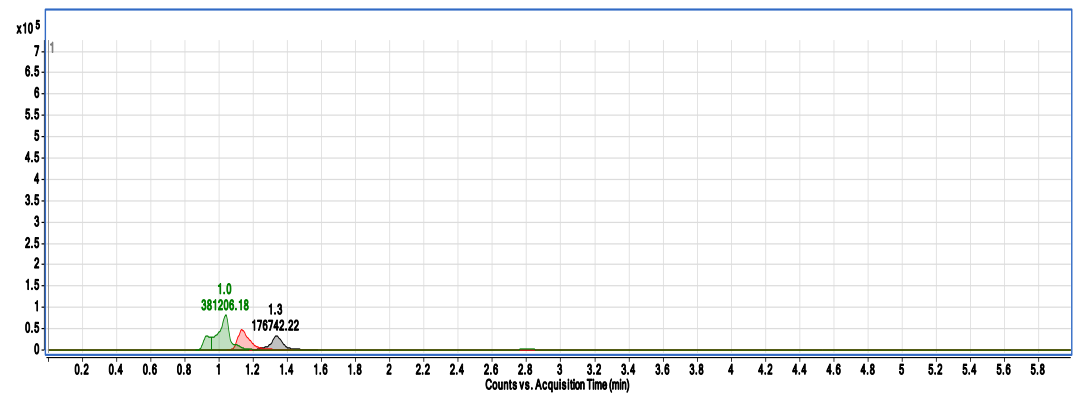

Figure 2. Original chromatography for the three catecholamines.

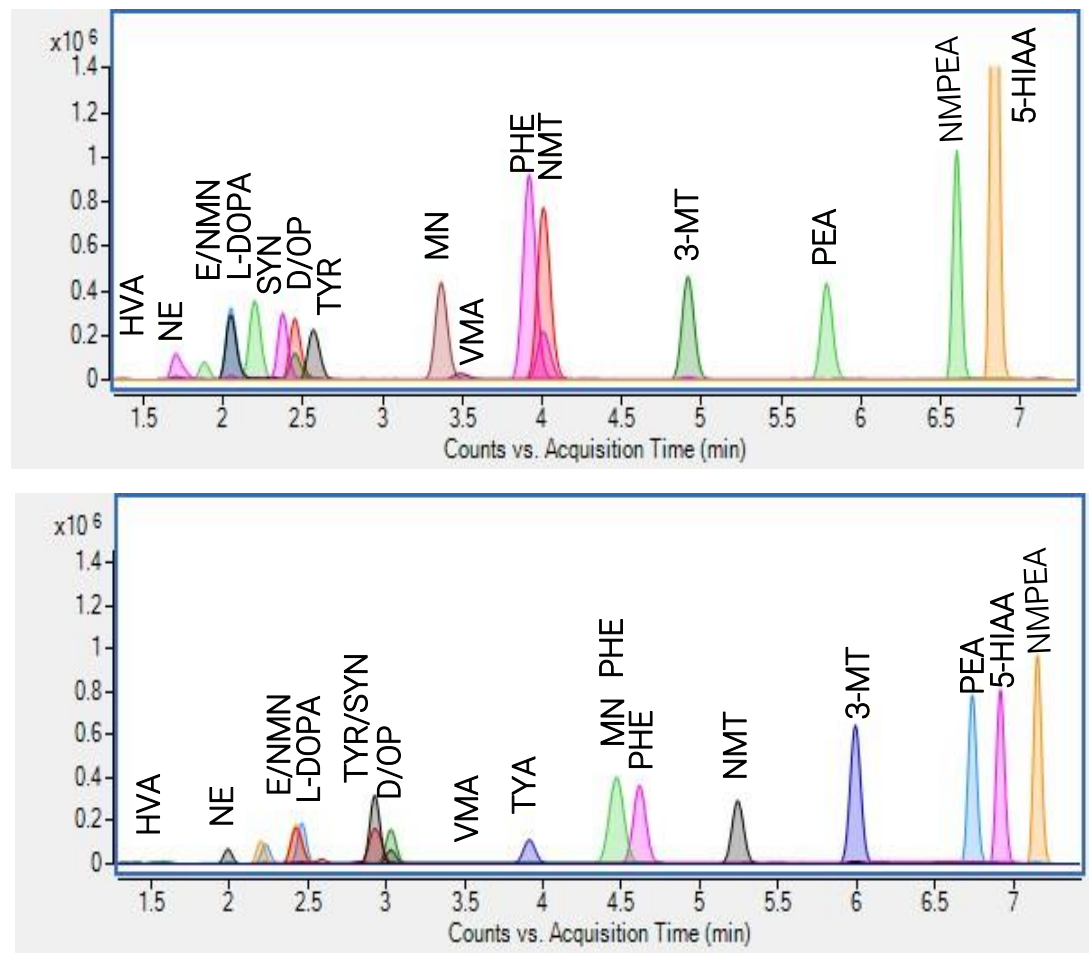

Figure 3. Example chromatograms with 18 analytes in 9.5 minutes using standard RP regime on a C18 column (top) and including ion pairing reagents (bottom).

Method Development

Previous work had demonstrated excellent sensitivity for the catecholamines, but these methods often require large sample volumes and include a significant concentration step before injection. Prior to addressing the sample prep portion of the workflow, experiments were undertaken to optimize the chromatographic resolution of the catecholamines from each other, as well as from their metabolites and precursors in this extended pathway study. A variety of ion pairing reagents were tested to determine where the best balance was between separation of isobars and sensitivity of analytes. Reagents examined covered a range of sulfonic acids, including butane (BSA), hexane (HxSA), heptane (HpSA), and octane (OSA). The chromatograms from these experiments are shown in Figure 4.

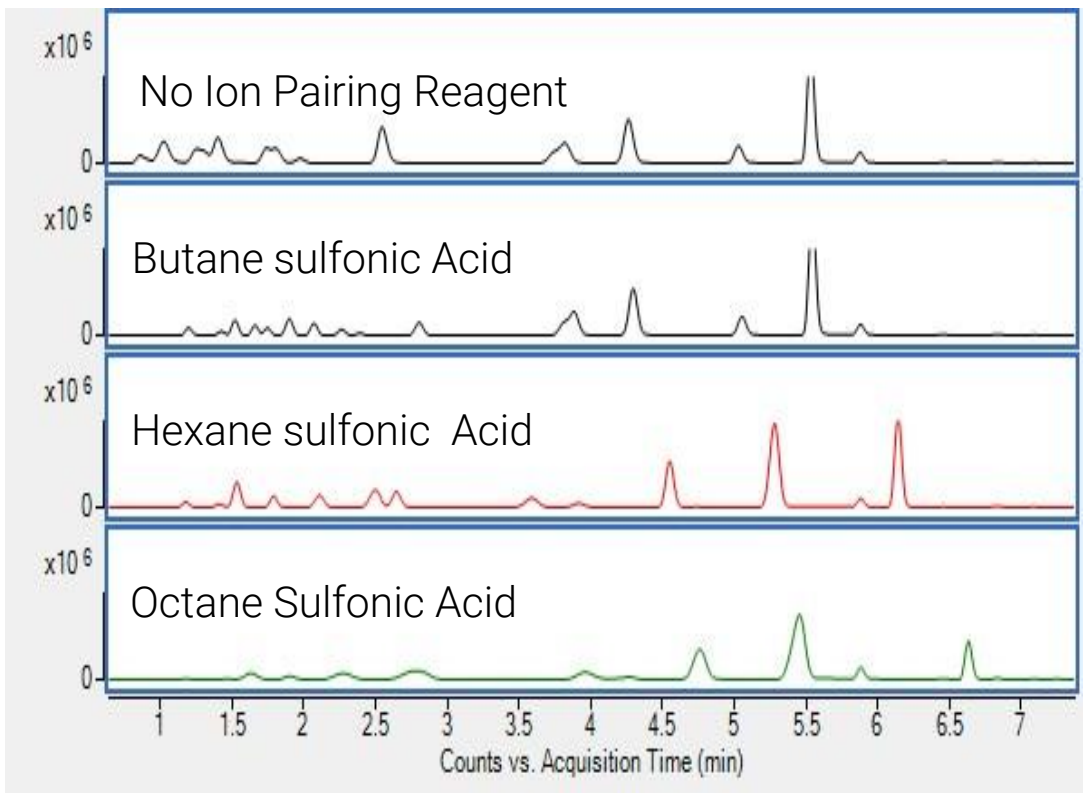

Figure 4. Chromatograms showing separation with addition of different ion pairing reagents to the vial. No reagent, BSA, HxSA, HpSA, and OSA are shown in order.

The ion pairing reagents were added directly to the injection vials, rather than to the mobile phase, which had the benefit of minimizing system contamination. In fact, the ion pairing reagent could be completely flushed from the system in about an hour (shown in Figure 5), which easily allows for method switching if the system is utilized for a variety of assays.

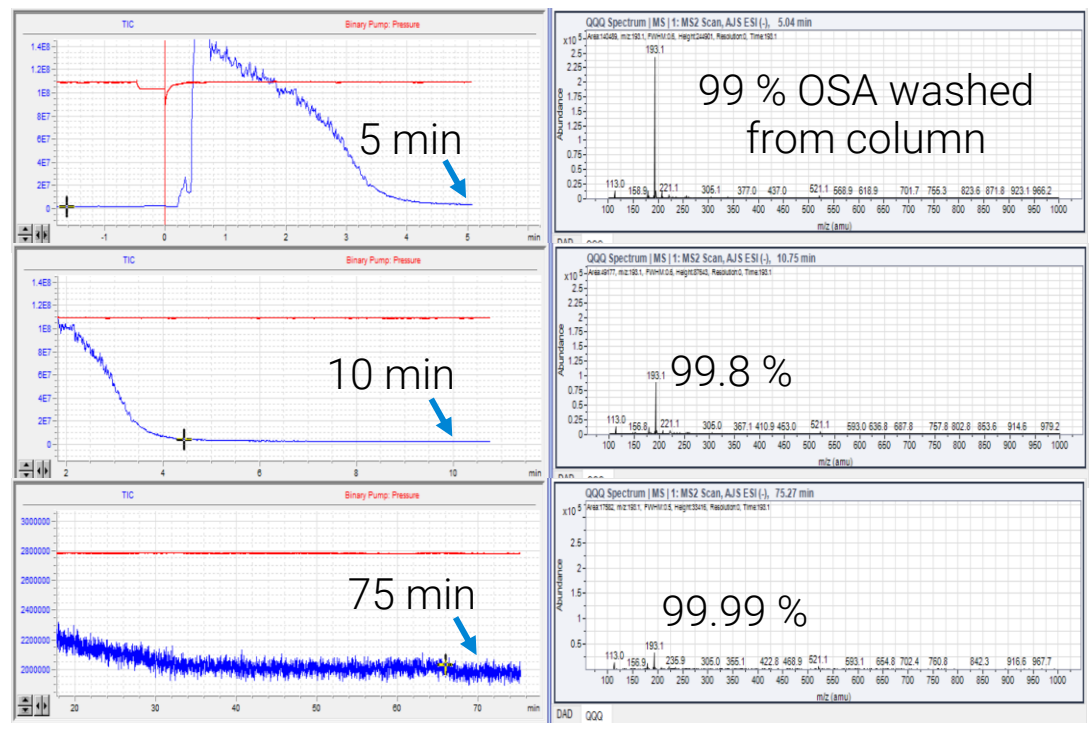

Figure 5. Background check for system contamination following LC flush after ion pairing injection.

It was found that HSA (hexane) was the optimal choice out of all the reagents tried when balancing retention enhancement with suppression of the early eluters. Attempts were made to utilize the original sample prep protocol featuring the Bond Elut PBA SPE cartridges; however, after some trial and error with samples it was found that the chemistry of the packing material was not compatible with the structures of many of the additional compounds, leading to non-detection of those analytes. Due to this hiccup, future work will focus on derivatization reagents to enhance signal, as well as to remove any isobar concerns.

Conclusions

The analysis of the extended plasma catecholamines pathway is complicated by several factors, including the sensitivity requirements, as well as the typically low sample volumes. In an attempt to address these issues, a novel method was developed to enhance the separation of isobars without adding significant complexity to the sample preparation procedure. However, the early eluting analytes were suppressed by the ion pairing reagents, so this method may not be suitable when sensitivity is the primary concern.

References

Analysis of Catecholamines in Urine by Unique LC/MS Suitable Ion-pairing Chromatography - Marianne L. Bergmann, et al., J of Chromatography B, April 2017.

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