Chiral Separation and Analysis of Methylphenidate, Ethylphenidate and Ritalinic Acid in Blood by Liquid Chromatography/Mass Spectrometry (LC/MS/MS)

Christina R. Smith, BS*; Marlon Young, BS; Madeleine J. Swortwood, PhD

Department of Forensic Science
Sam Houston State University
Huntsville, TX, USA

FSF Emerging Forensic Scientist Award Competition

FSF Emerging Forensic Scientist Award
Paper Presentation

Disclaimer

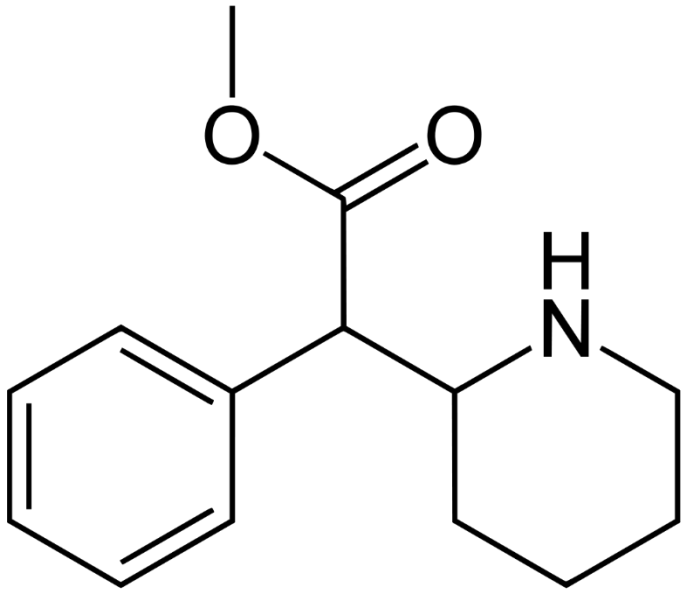
- The authors have nothing to disclose at this time.

Attention-deficit/hyperactivity disorder (ADHD)

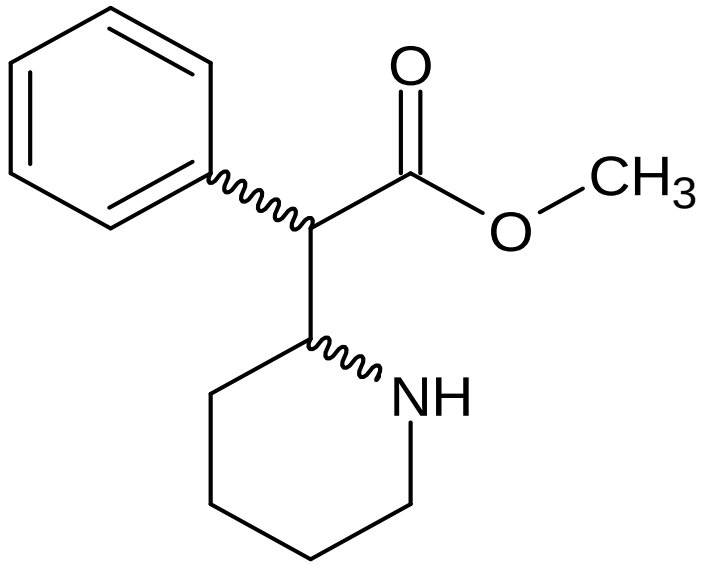
- Neurobehavioral disorder
  - Boredom, difficulty hearing and listening
- Lack of focus
  - Due to: inattention, hyperactivity, impulsivity

Methylphenidate (MPH)

- Most commonly prescribed ADHD medication
- Speeds up brain activity
- 1990s – psychostimulant use, recreational abuse
- Schedule II – medical use, high potential for abuse



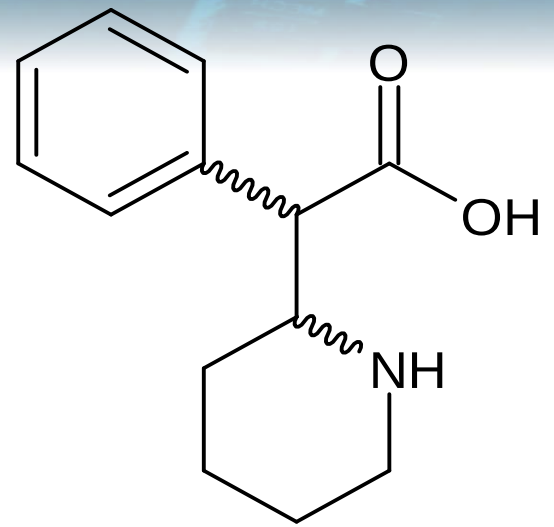
MPH Metabolism



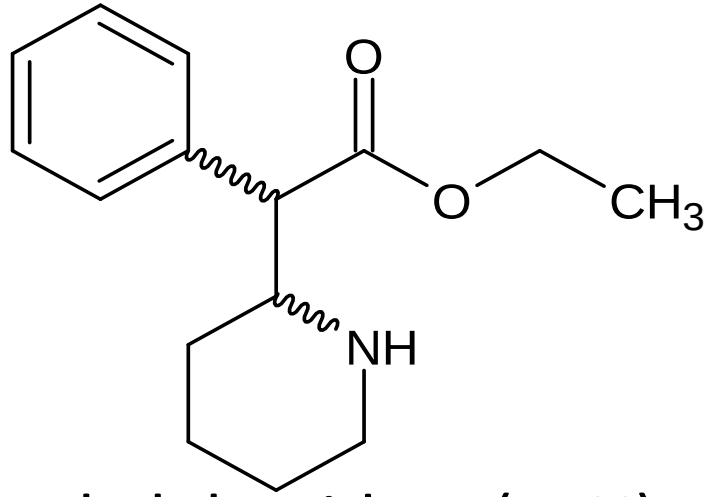
Methylphenidate (MPH)

CES1

+EtOH



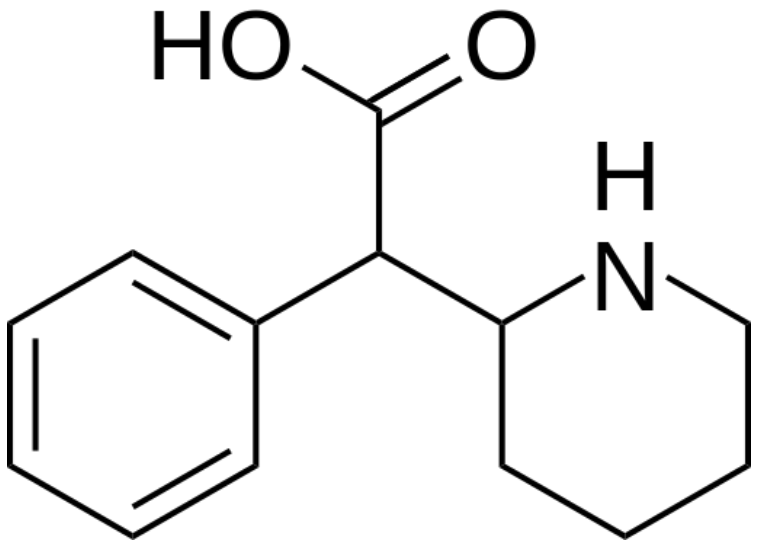
Ritalinic Acid (RA)



Ethylphenidate (EPH)

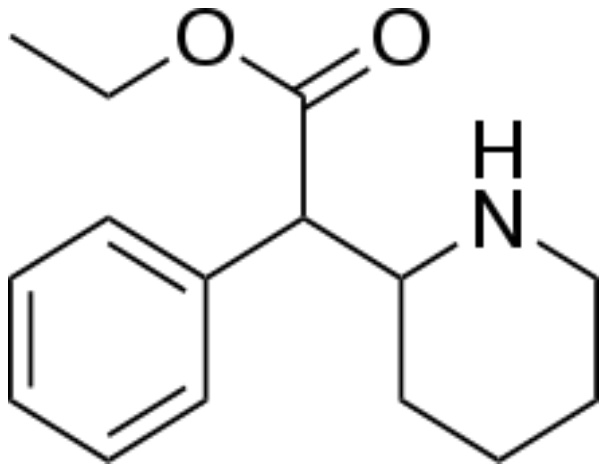
Ritalinic Acid

- Inactive metabolite of MPH
- Structurally different from MPH
  - Carboxylic acid group
  - Poses a challenge for analytical extraction



Ethylphenidate

- MPH and alcohol co-abuse
- Case studies: 2 fatal overdoses



- More recently: abused alone
  - Purchased over internet

[–] **Borsbakenare** 4 points 8 months ago

I have used ethylphenidate multiple times to study.

It works like a charm for long hours behind the desk. I got really motivated to get shit done and enjoyed the process of studying.

Compared to methylphenidate it's more euphoric IME.

I stopped using this chemical due to the harsh comedowns, i really hate that

After studying, you feel completely empty and the next day i could feel my heart ache sometimes.

Purpose of this study

- To optimize and fully validate a method for the chiral separation and quantitation of the *l/d* enantiomers of threo-MPH and EPH as well as RA in blood using liquid chromatography coupled with mass spectrometry

Previous Studies (Chiral)

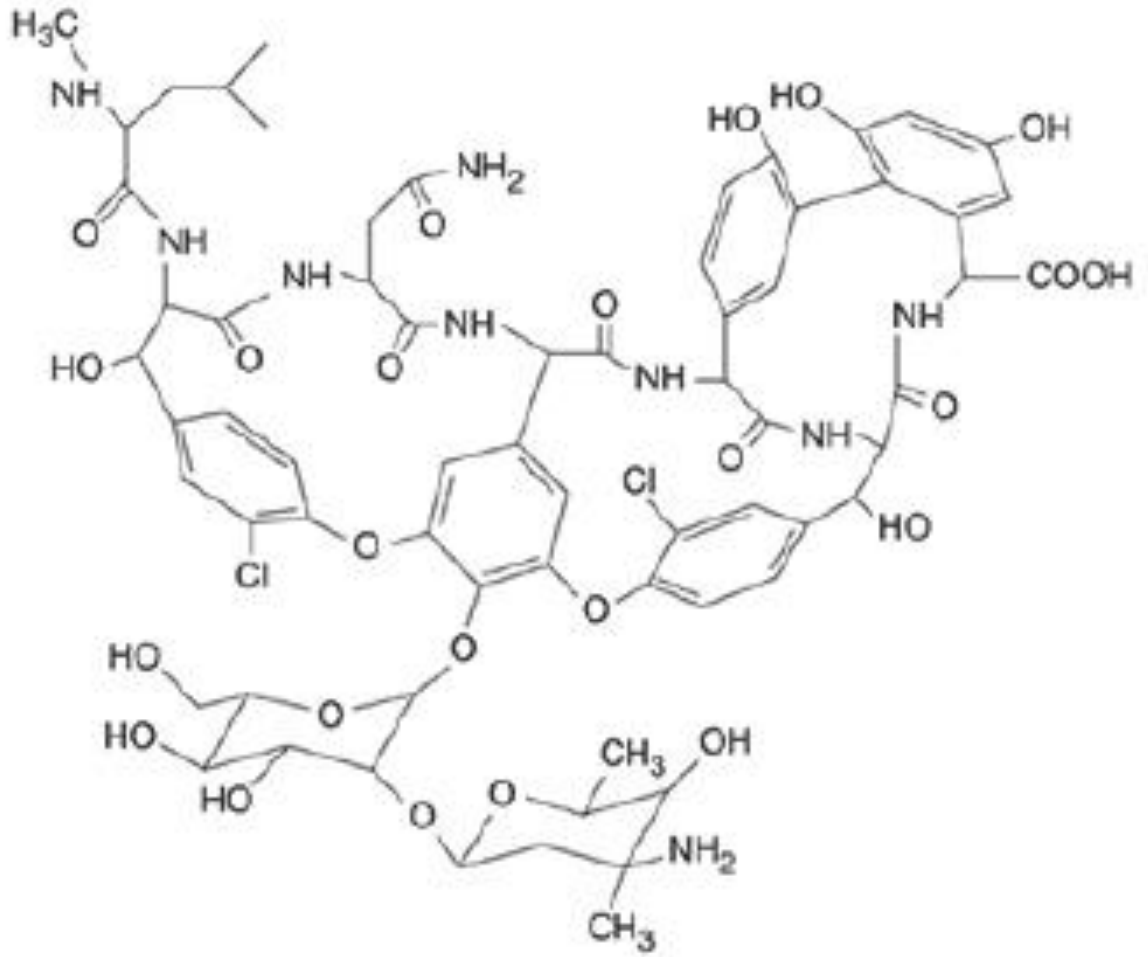
Author	Analytes	Matrix	Extraction	Instrumentation	Chiral Technique	LOQ
Ramos (1999)	MPH	Plasma	LLE	LC/APCI-MS/MS	Column	0.087 ng/mL
Zhu (2011)	MPH, EPH	Plasma	LLE	LC-MS/MS	Column	0.025 ng/mL
Thomsen (2012)	MPH, RA	Blood	PP, SPE	LC-MS/MS	Column	0.5 ng/g
Combs (2013)	MPH	Mouse Brain	SPE	LC-MS/MS	Column	7.5 ng/mL

Objectives

- To develop and optimize an extraction method for MPH and its metabolites from blood
- To separate the enantiomers of MPH and EPH with RA using liquid chromatography
- To validate a method for MPH and its metabolites to be quantified using tandem mass spectrometry

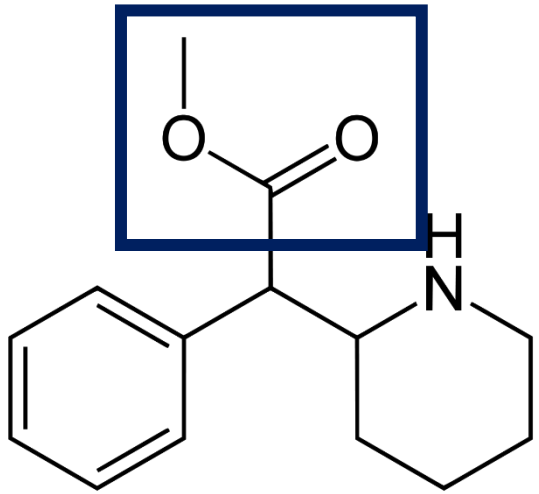
Method Development

- Column Selection
  - Previous literature
    - Astec Chirobiotic V2
    - Chiral AGP
  - This method:
    - Agilent InfinityLab Poroshell Chiral-V
    - Chiral selector: Vanomycin



Method Development

- Extraction
  - Varying groups on the structures poses analytical challenge
  - Attempted SLE, LLE, SPE to recover all analytes



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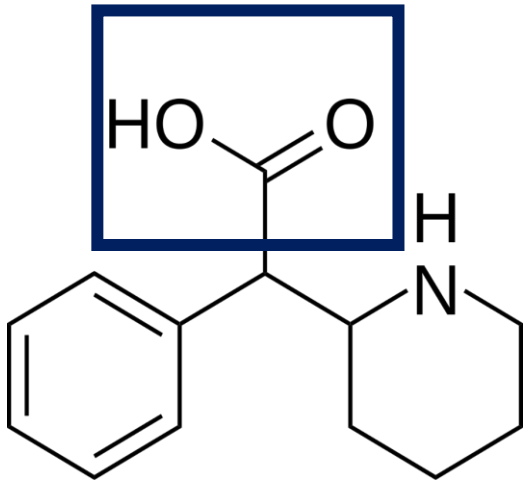

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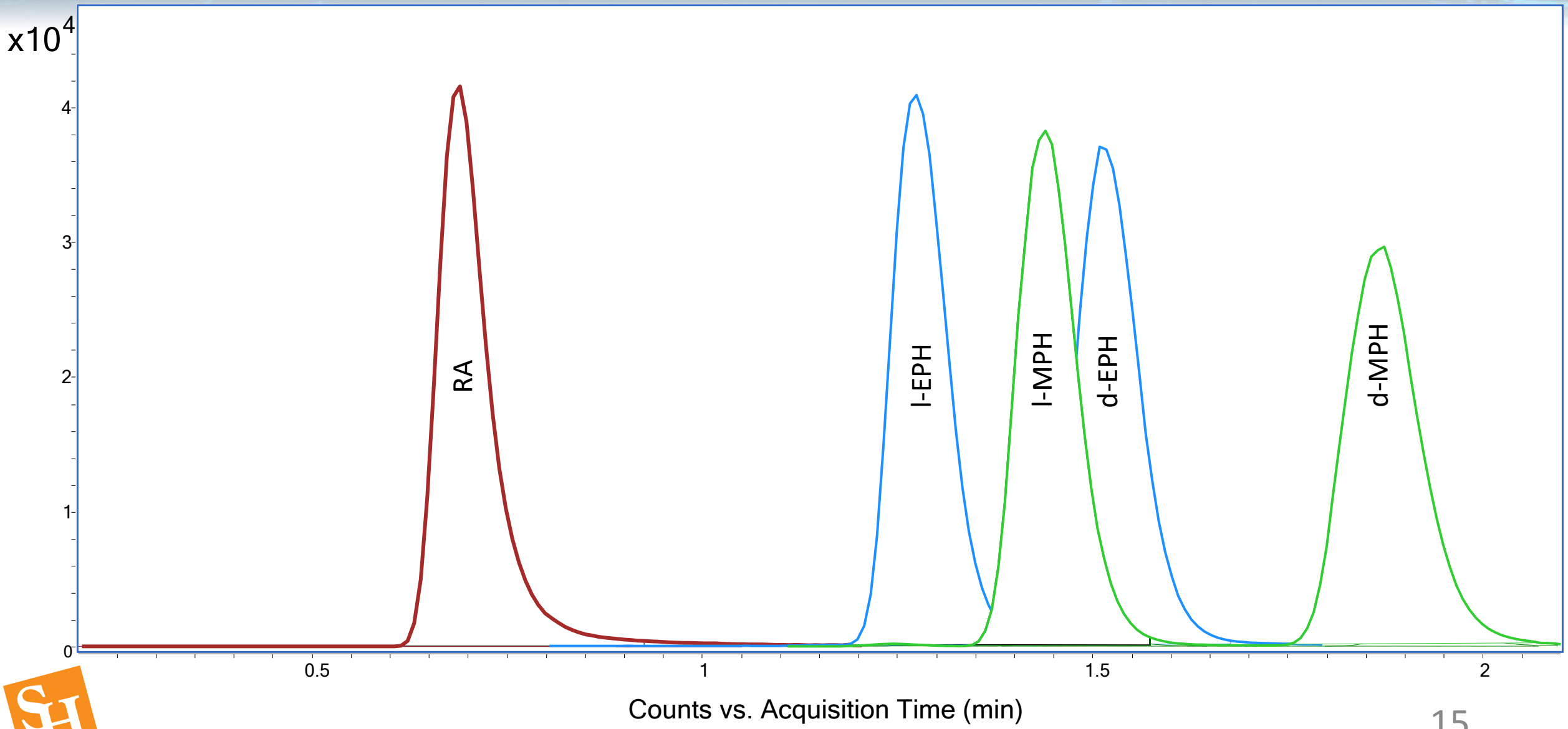
  - Use of UCT DAU column solved the problem

Final Parameters

Agilent Technologies 1290 Infinity LC	
Column	Agilent Poroshell Chiral-V (2.1 x 100mm x 2.7 µm)
Mobile Phase	A: di H2O B: 0.025% Ammonium acetate with 0.0125% TFAA in MeOH
Flow Rate	0.600 mL/min (isocratic – 2% A, 98% B)
Agilent Technologies 6470 Triple Quadrupole MS	
Ionization Type	Electrospray ionization (ESI) positive
Acquisition Mode	MRM
Injection Volume	1 µL

EIC


x10^4
4
3
2
1
0
RA
l-EPH
l-MPH
d-EPH
d-MPH
0.5
1
1.5
2
Counts vs. Acquisition Time (min)

Extraction

Dry	• 25 µL of internal standards and drugs
Add	• 250 µL of blood • 100 mM phosphate buffer (1 mL)
Wait	• Vortex • Let stand for 5 minutes
Centrifuge	• 2000 rpm, 10 minutes
Condition	• 1mL Methanol (1 mL)
Condition	• 100mM phosphate buffer (1 mL)
Apply	• Sample to column (DAU Clean Screen, 130mg)
Wash	• 100mM acetic acid (1 mL)
Wash	• MeOH (1 mL)
Dry	• With pressure, 10min
Elute	• 2% NH4OH in MeOH (2 mL)
Dry	• Evaporate to dryness under nitrogen
Recon	• Mobile phase (2:98) (100 µL)

Method Validation

- ANSI/ASB Standard 036:
- Quantitative Validation
  - Linearity
  - Limit of Detection (LOD)
  - Limit of Quantitation (LOQ)
  - Bias and Precision
  - Matrix Effects
  - Interference
  - Carryover
  - Dilution
  - Stability

Linearity, LOD, LOQ

Analyte	LOD	LOQ	Linear range (ng/mL)	R^2 (mean, n=5)	Weighting
l-MPH	0.1	0.5	0.5-200	0.999	1/x
d-MPH	0.1	0.5	0.5-200	0.998	1/x
l-EPH	0.1	0.5	0.5-200	0.999	1/x
d-EPH	0.1	0.5	0.5-200	0.998	1/x
RA	0.5	0.5	0.5-500	0.999	1/x

Bias and Precision

- 3 concentrations
  - MPH and EPH – 1.5, 25 and 150 ng/mL
  - RA – 1.5, 25 and 400 ng/mL
- Run over 5 days in triplicate (n=15)

Analyte	Bias, %			Between Run Precision, %CV			Maximum Within Run Precision, %CV		
	LQC	MQC	HQC	LQC	MQC	HQC	LQC	MQC	HQC
L-MPH	-10.0	-7.9	-8.9	5.5	4.1	4.9	7.5	4.9	7.6
D-MPH	-10.7	-6.4	-8.6	6.0	5.6	5.2	8.9	5.2	8.9
L-EPH	-12.7	-10.3	-10.3	4.5	3.6	4.8	8.7	4.7	7.3
D-EPH	-11.5	-7.1	-10.2	6.8	6.9	5.9	12.5	5.6	9.4
RA	-9.5	-4.8	-7.1	5.6	5.0	6.1	5.4	9.1	4.7

Matrix Effects

- Matrix effects analyzed by post-extraction addition

Analyte	Matrix Effects, % (n=10)	
	LQC	HQC
l-MPH	44.5	20.4
d-MPH	24.0	4.8
l-EPH	44.6	21.6
d-EPH	48.7	24.2
RA	-48.3	-55.6
l-d10-MPH	44.7	19.5
d-d10-MPH	28.6	4.8
d10-RA	-47.2	-55.7

Interference

- Endogenous, exogenous and common drugs were tested for interferences
- No blood blanks or negative controls had the presence of any analytes
- LQC was spiked with drug mixes
  - LQC still quantified within accuracy (maximum bias -20%) with no interfering peaks

Stability, Carryover and Dilution

Validation Parameter	Results
Autosampler Stability	-16.6 to -6.0% (bias)
Room Temperature Stability	-54.7 to 35.5% (bias)
Carryover	<LOD
Dilution (1:10)	-13.0 to 5.5% (bias)

Conclusion

- This method developed and optimized an extraction method for MPH, EPH and RA
- The enantiomers of MPH and EPH were fully separated utilizing LC and a chiral column
- This is the first known method to quantify the enantiomers of MPH and EPH in addition to RA in a single assay

Acknowledgements

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Questions?

Christina Smith
Doctoral Student

crs040@shsu.edu

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