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HRMS Bioanalysis for Fast PK

Accelerating Lead Optimization

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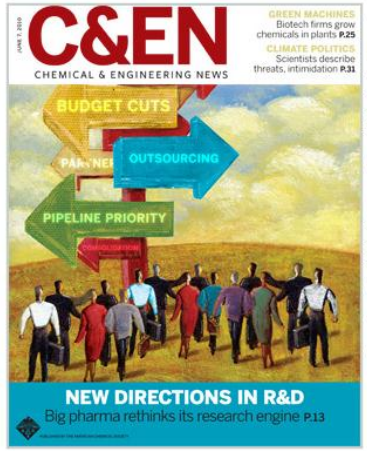
Introduction

- Advantages of DBS in Discovery
  - Can it be used in a generic manner?
- HRMS bioanalysis– a new tool?
- DBS case study using Verapamil
- Conclusions
  - Designing a better NCE…



June 7, 2010 Cover, Vol. 88, Issue 23

Research Recalibrated

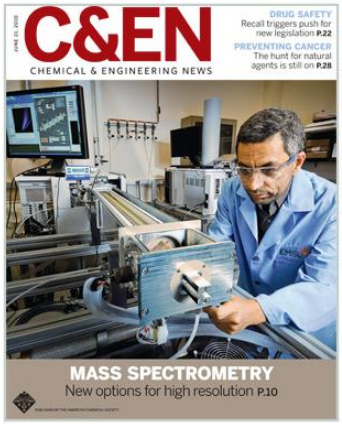
The spending race is over in pharmaceutical R&D, with companies now focused on doing more with less. (pp. **13-18**)

COVER: Veer

R&D On The Chopping Block pp. 14-15

Big pharma's path through the recession is littered with job and program cuts and plant closures.

» Full Article



June 21, 2010 Cover, Vol. 88, Issue 25

High-Res Mass Spec

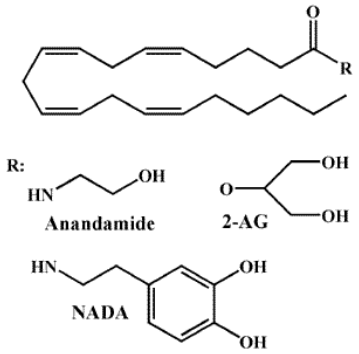
Instruments with increased resolving power and accuracy give users more choices than ever before. (pp. **10-15**)

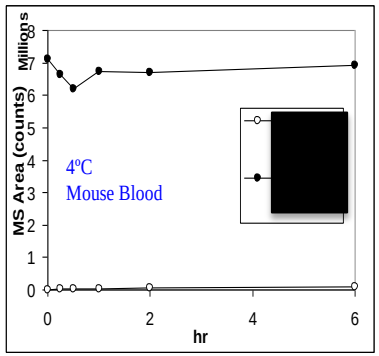
COVER: Yehia Ibrahim is part of a team that is coupling ion mobility with time-of-flight mass spectrometry for high-resolution proteomics measurements at Pacific Northwest National Laboratory's Environmental Molecular Sciences Laboratory. Scott Butner/PNNL

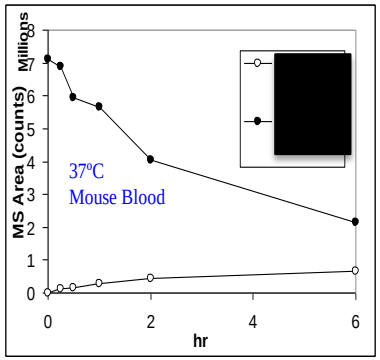
» Full Article

Advantages of DBS in Discovery PK

- Compound stability is less of a concern
  - *Time & temperature impact between blood draw & plasma harvesting*
  - Esterases & Amidases (hydrolases) add water to the substrate with no additional cofactors needed. While enzymatic hydrolysis reaction is irreversible, the action of esterases and amidases to form two products (acid and alcohol or amine) can be *reversible*.
  - When the clearance is greater than cardiac blood flow, it indicates that the compound is being cleared by the blood or by blood vessels.







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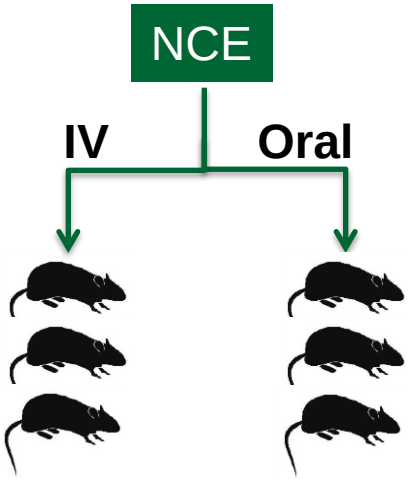
Advantages of DBS in Discovery PK

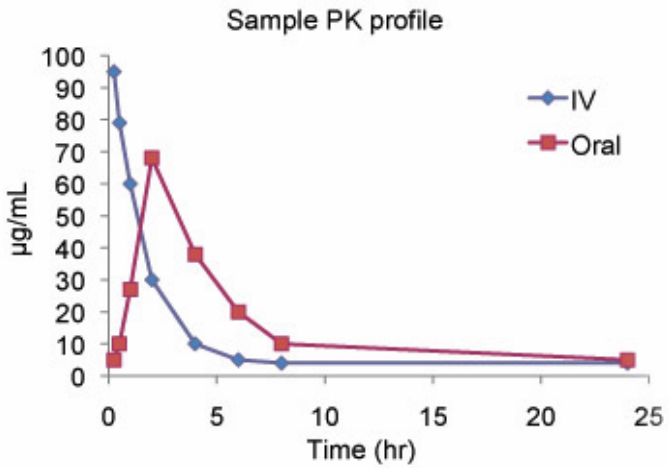
- Passive sampling possible
- Micro-sampling
  - *Small blood draws reduce stress related physiological changes that impact levels of RBC/WBC/Platelets, cortisol, corticosterone, prolactin, etc; factors that could impact PK data*

Body weight (g)	*CBV(ml)	1% (ml)	10% (ml)
20	1.10 - 1.40	.011 - .014	.11 - .14
25	1.37 - 1.75	.014 - .018	.14 - .18
30	1.65 - 2.10	.017 - .021	.17 - .21
35	1.93 - 2.45	.019 - .025	.19 - .25
40	2.20 - 2.80	.022 - .028	.22 - .28
125	6.88 - 8.75	.069 - .088	.69 - .88
150	8.25 - 10.50	.082 - .105	.82 - 1.0
200	11.00 - 14.00	.11 - .14	1.1 - 1.4
250	13.75 - 17.50	.14 - .18	1.4 - 1.8
300	16.50 - 21.00	.17 - .21	1.7 - 2.1
350	19.25 - 24.50	.19 - .25	1.9 - 2.5

*Circulating blood volume

Rodent Fast PK: Proven Model for Human PK





Fast PK Metrics

- Exposure route
  - Oral vs IV?
- Speed
  - Quantitation with quality
  - Peptides or small molecules

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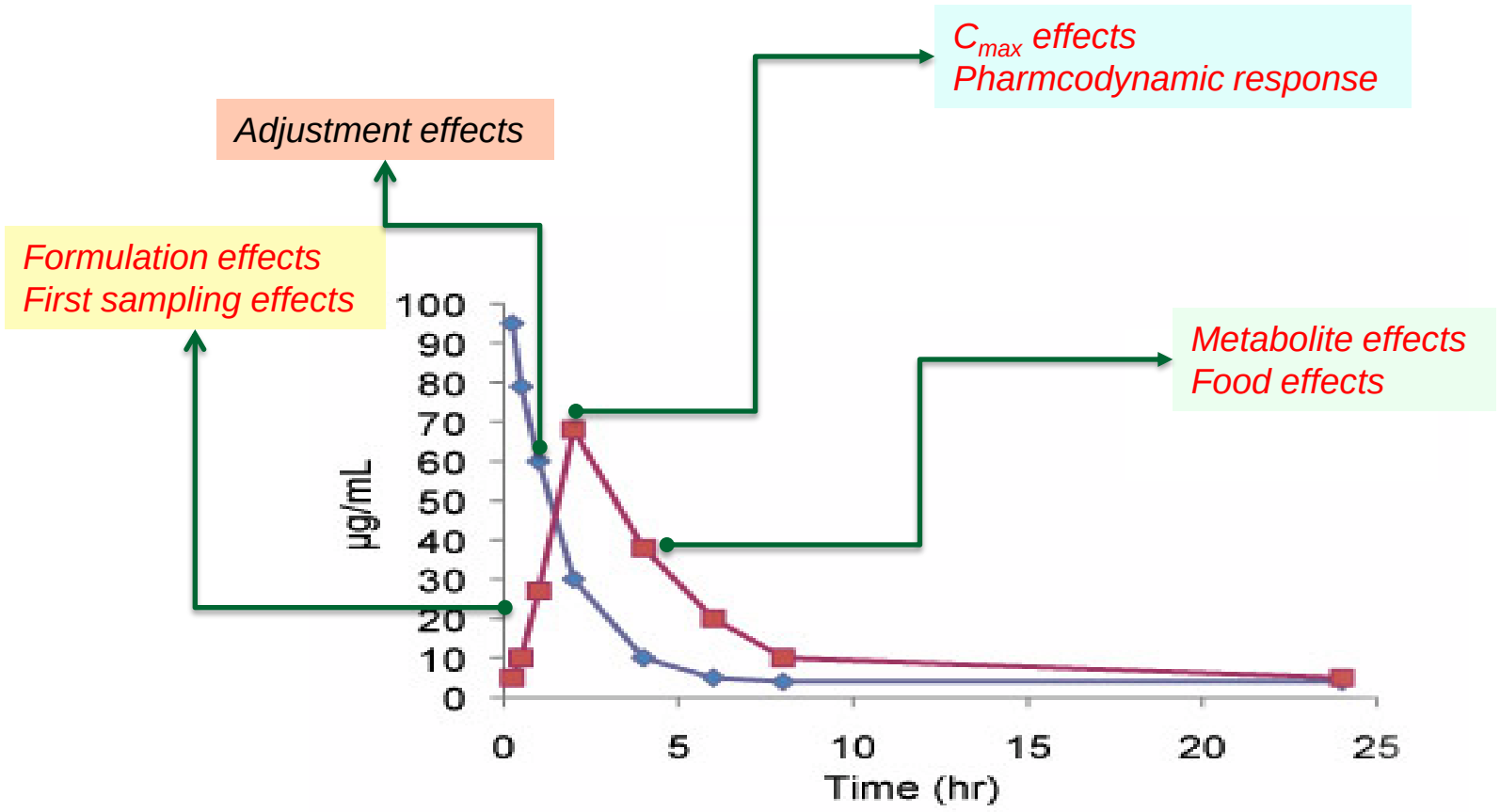
Maximize Information Content



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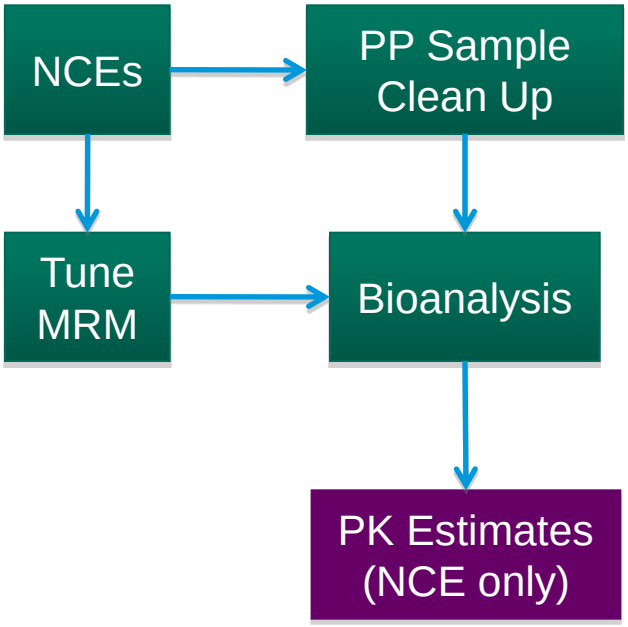
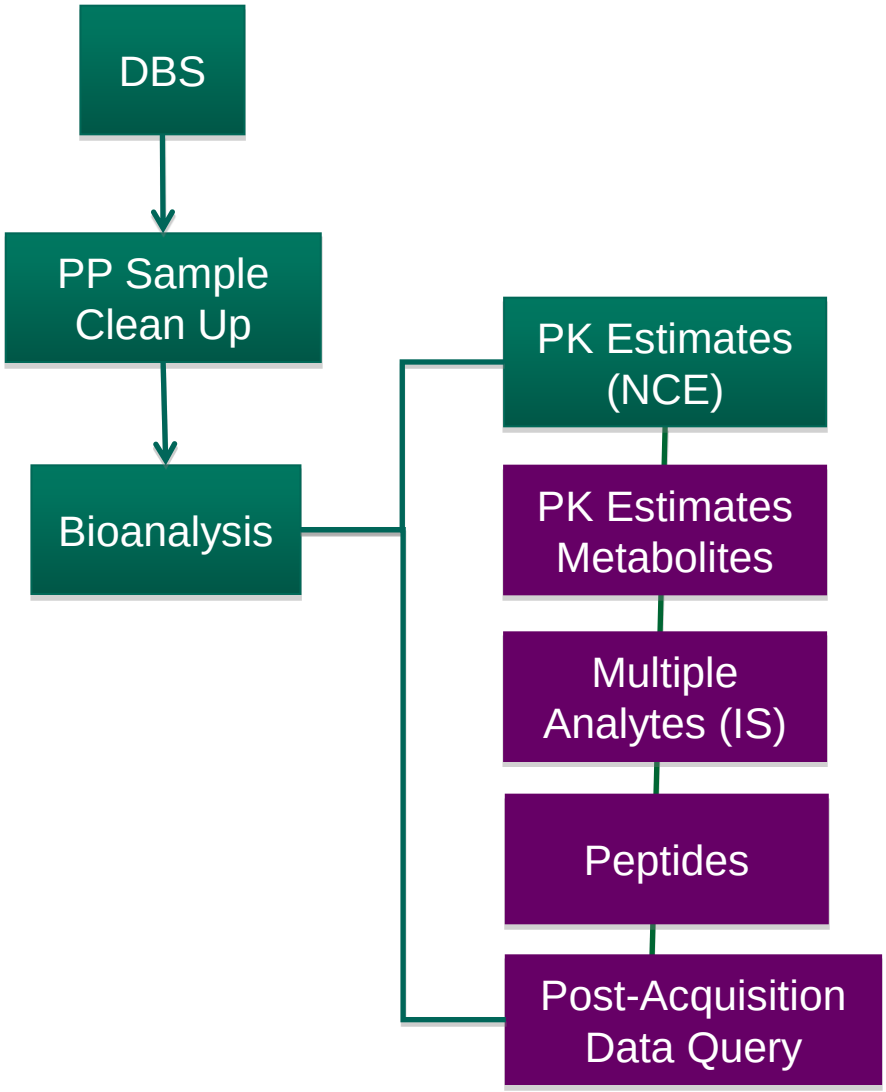
Traditional Workflow (MRM)



LC-MRM Analysis

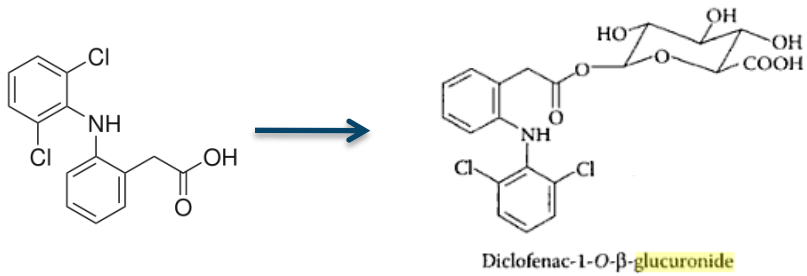
- Tuning MRM **takes time** & requires some level of expertise
- **Peptides** require determination of charge state and the optimal MRM transition
- Difficult to get metabolite information
- Limited information from sample
  - NCE PK only – *no other information from incurred sample*

DBS & HRMS – Critical New Value (?)



HRMS Bioanalysis

- No compound dependent tuning required – easier to use/faster to set-up
- Full Scan Peptide Bioanalysis (no MRM)
- Post-acquisition data analysis
- Providing PK data as well as *critical new information (metabolites: acyl-glucoronides?)*



Diclofenac-1-*O*-β-glucuronide

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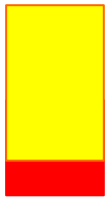
Challenges

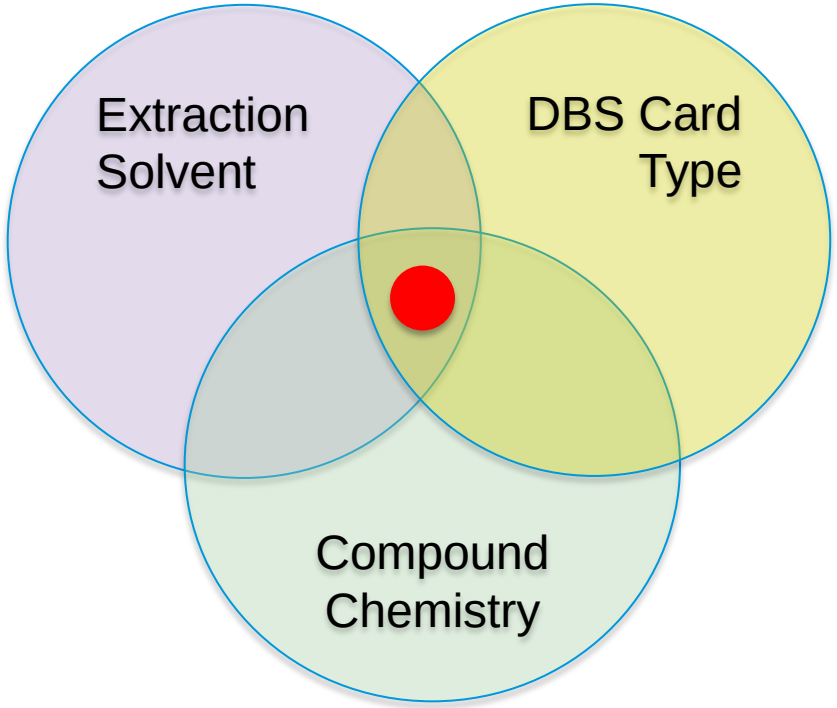
- Bioanalytical
  - Sensitivity (1ng/mL)
  - Recovery using a generic extraction protocol
  - Dilution & Re-assay
  - Automation & Discovery time-lines (turn-around time)
- Sampling
  - Type of card
  - Sample volume (15 v 25uL)
  - Blood-plasma ratio (BPR will influence PK)
    - > *Tempting to assume even distribution*
    - > *Be aware of concentration dependent partitioning*

Even distribution in RBC (PCV)

Extensive distribution in RBC (PCV)



Limited distribution in RBC (PCV)

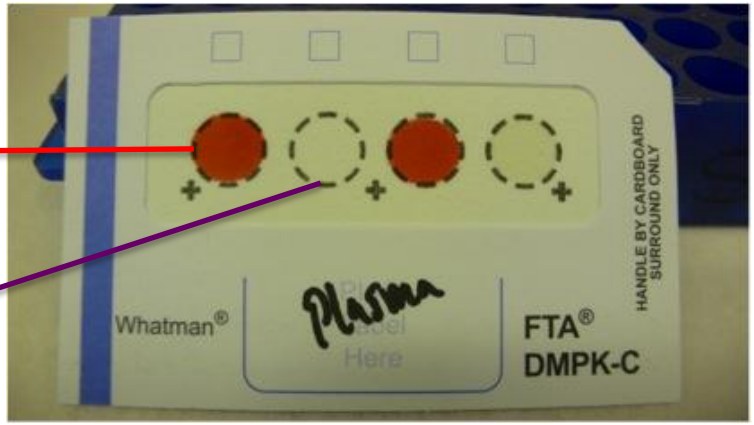


DBS

BASIC EXPERIMENTS

Spot Visualization

- Spot visualization (upon drying) – 1 DBS card per animal?
- Spotting volume – how much?
- Hematocrit – impact on blood viscosity & spot diffusion
- Recovery – which extraction solvent to use?
- Ion suppression from different cards



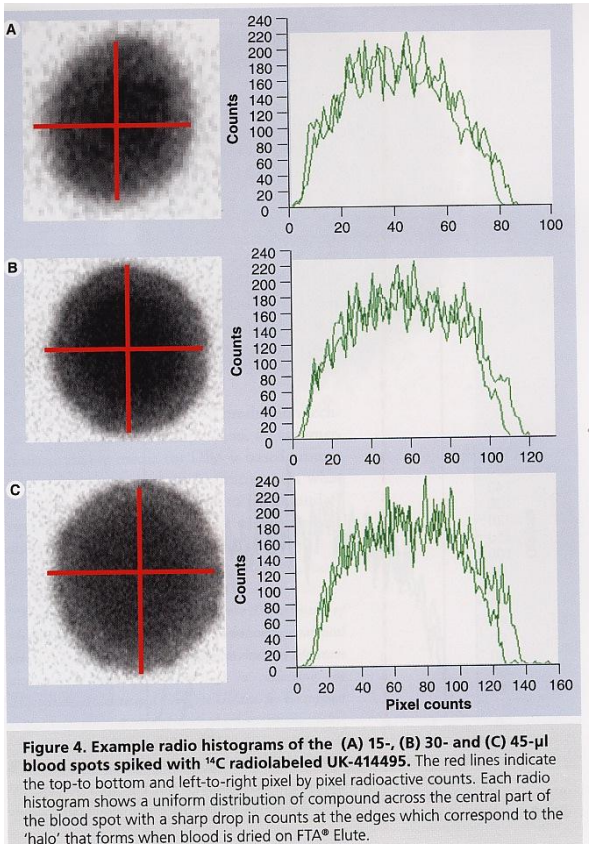


Figure 4. Example radio histograms of the (A) 15-, (B) 30- and (C) 45-µl blood spots spiked with ^{14}C radiolabeled UK-414495. The red lines indicate the top-to bottom and left-to-right pixel by pixel radioactive counts. Each radio histogram shows a uniform distribution of compound across the central part of the blood spot with a sharp drop in counts at the edges which correspond to the 'halo' that forms when blood is dried on FTA® Elute.

Spot Visualization

Utilization of DBS within drug discovery: development of a serial microsampling pharmacokinetic study in mice: Clark et al., 2010. Bioanalysis 2 (8), 1477-1488

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Spot Diffusion

- Diffusion ≈ viscosity (capillary effect) ≈ hematocrit
- Conclusion 1: use smallest volume possible
- Conclusion 2: use 1 DBS card per time-point ($4/card!)

Plasma Spot (*25uL*)

Urine Spot (*25uL*)

MeOH:H2O (*25uL*)
(90:10, v/v)

DBS Generic Extraction Solvent – (90:10, MeOH-Water, v/v)

Procedure	
Stock Std	50ug/mL of 15 compounds in ACN:MeOH @9:1
Blood @1,000 ng/mL	Mix 20 uL of Stock Std and 980 uL of Human Blood
Spiking @100ng/mL	Mix 20 uL of Stock Std with 9.980 mL of MeOH:DMSO:H2O @7:1:2

Sample Preparation	
1	Spot 25uL of blood spiked with 1ug/mL std cocktail (Spot diameter ~ 8 mm)
2	After 2 hrs, punch a 6 mm spot
3	Add 100 uL of MeOH:H2O @9:1. Vortex for 30 min
4	Transfer 50 uL of solution into a new tube
5	Evaporate to dryness
6	*add 300 uL of MeOH:H2O @9:1 containing an IS*

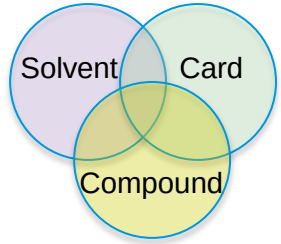
4000ng/mL

Control Preparation	
1	Spot 25uL of blood (blank) (Spot diameter ~ 8 mm)
2	After 2 hrs, punch 6 mm spot
3	Add 100 uL of MeOH:H2O @9:1. Vortex for 30 min
4	Transfer 50 uL of solution into a new tube
5	*Add 62.3 uL Spiking solution (100 ng/mL)*
6	Evaporate to dryness
7	*add 300 uL of MeOH:H2O @9:1 containing an IS*

4000ng/mL

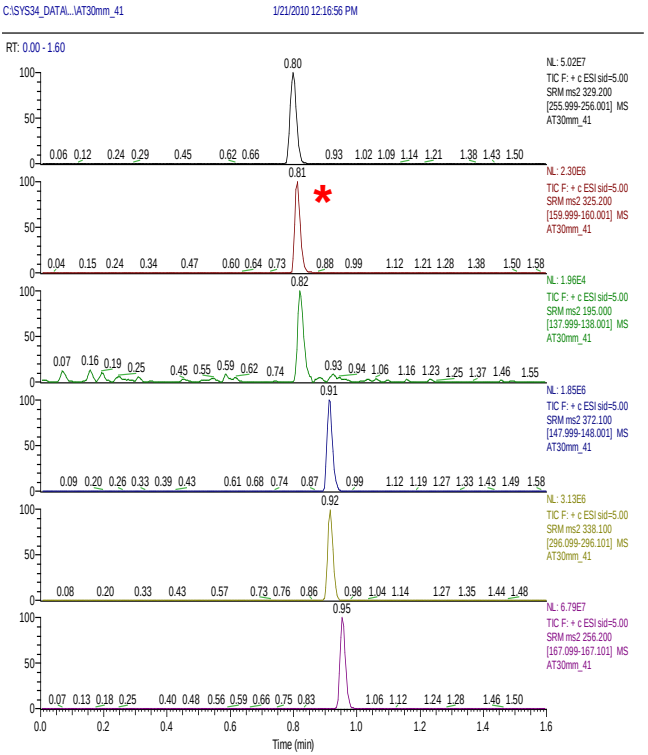
Recovery Results

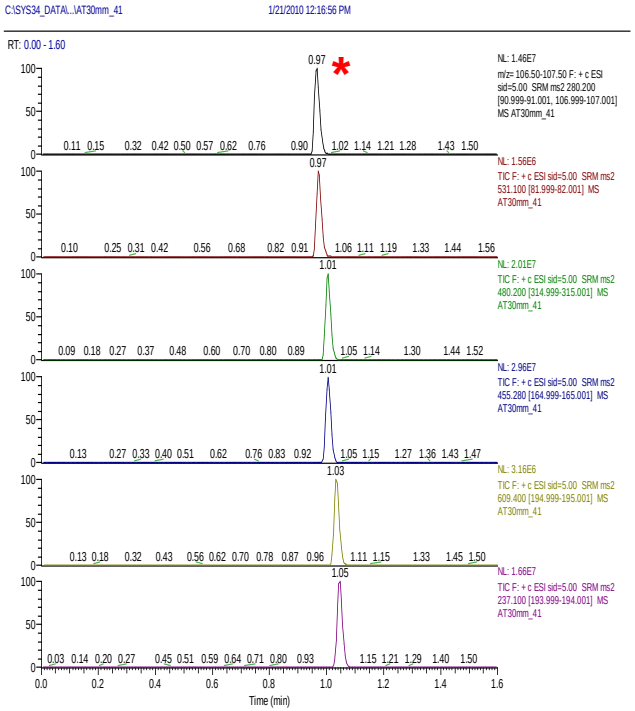
- Card used: Whatman DMPK-C
- Variable: compound hydrophobicity
  - > *Extraction time: **15min** on plate-shaker*

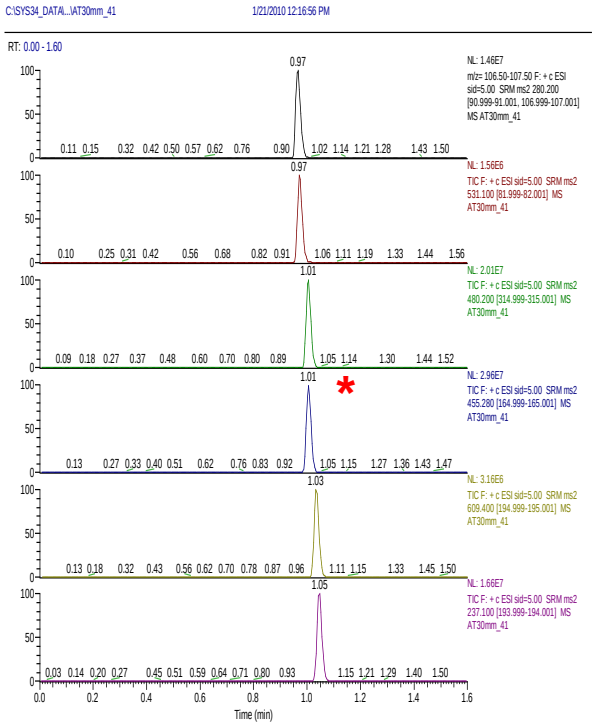


#	Name	Ret. Time	Formula	M+H	Recovery%
1	Tiapride	0.8	C15H24N2O4S	329.153	41.82
2	Quinine	0.81	C20H24N2O2	325.191	62.2
3	Caffeine	0.82	C8H10N4O2	195.088	
4	Trazodone	0.91	C19H22ClN5O	372.159	79.74
5	Linezolid	0.92	C16H20FN3O4	338.151	110.31
6	Diphenhydramine	0.95	C17H21NO	256.17	105.52
7	Doxepin	0.97	C19H21NO	280.17	99.05
8	Ketoconazole	0.97	C26H28Cl2N4O4	531.156	93.56
9	Nicardipine	1.01	C26H29N3O6	480.213	96.13
10	Verapamil	1.01	C27H38N2O4	455.29	86.39
11	Reserpine	1.04	C33H40N2O9	609.281	101.36
12	Carbamazepine	1.04	C15H12N2O	237.102	51.65
13	Chlorpromazine	1.04	C17H19ClN2S	319.103	96.57
14	Voriconazole	1.1	C16H14F3N5O	350.122	
15	Tolbutamide	1.15	C12H18N2O3S	271.111	92.41
16	Diazepam	1.18	C16H13ClN2O	285.079	106.56
17	Warfarin	1.27	C19H16O4	309.112	99.44

17 Internal Standards Test Compounds (Elution Profile)







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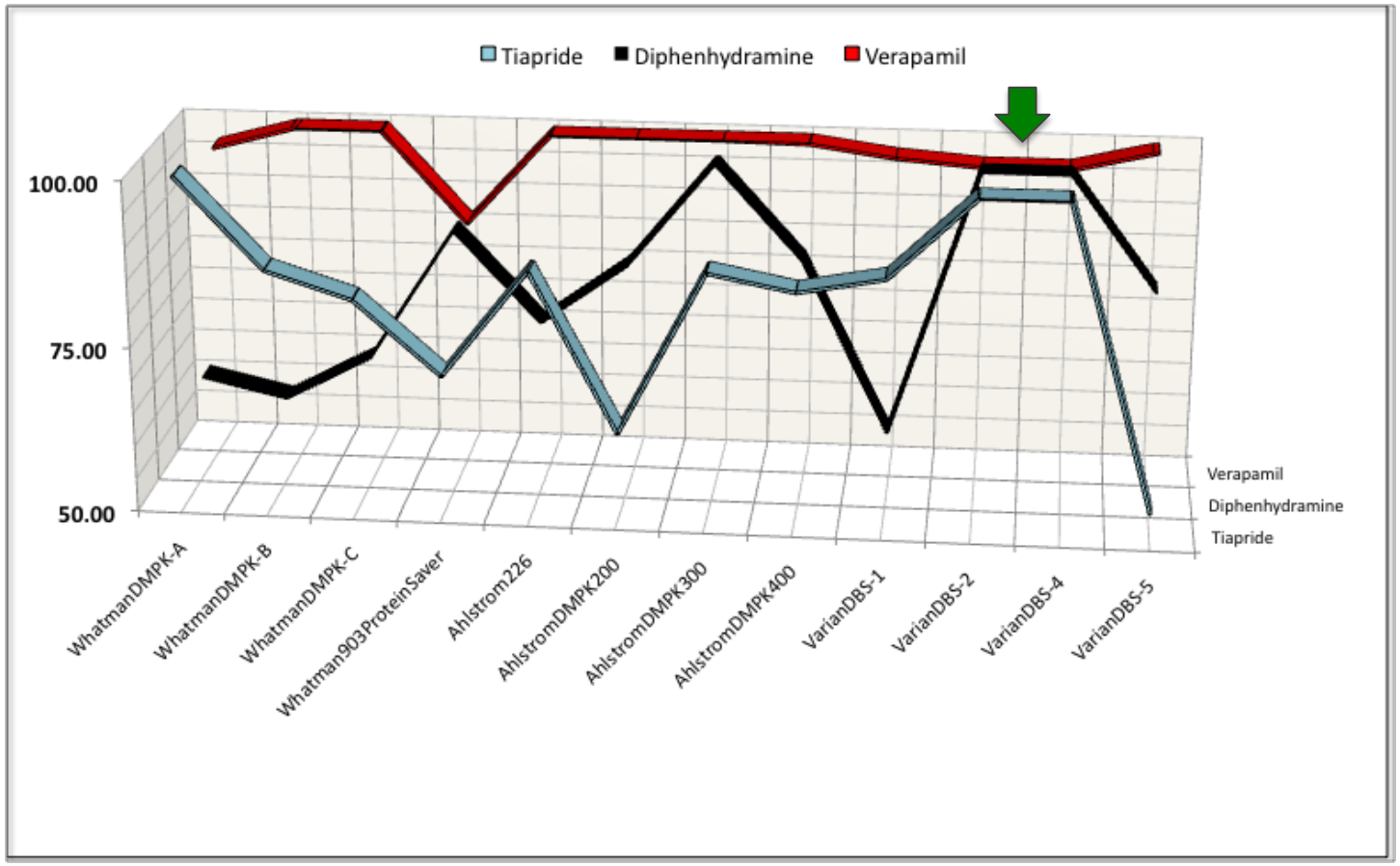
Recovery Results: DBS Card Types

- 3 Compounds chosen (Tiapride, Diphenhydramine, Verapamil)
- 12 different DBS cards tested for recovery using MeOH-Water (90:10, v/v)
  - *Extraction time: **30min** on plate-shaker*



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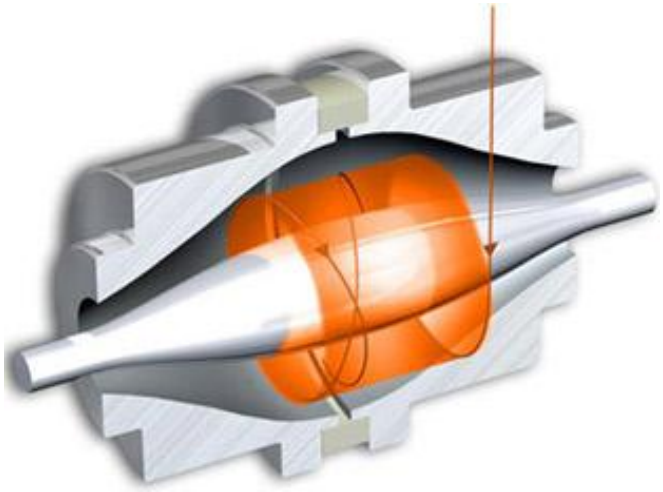

DBS

HRMS BIOANALYSIS

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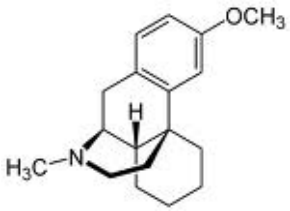
Power of Accurate Mass Measurements (Dextromethorphan)



Average Mass (M+H)

$C_{18}H_{25}NO$ = 272.4

Mono-isotopic (most abundant isotope of each element)

$^{12}C_{18}{}^{1}H_{26}{}^{14}N^{16}O$ = 272.2087

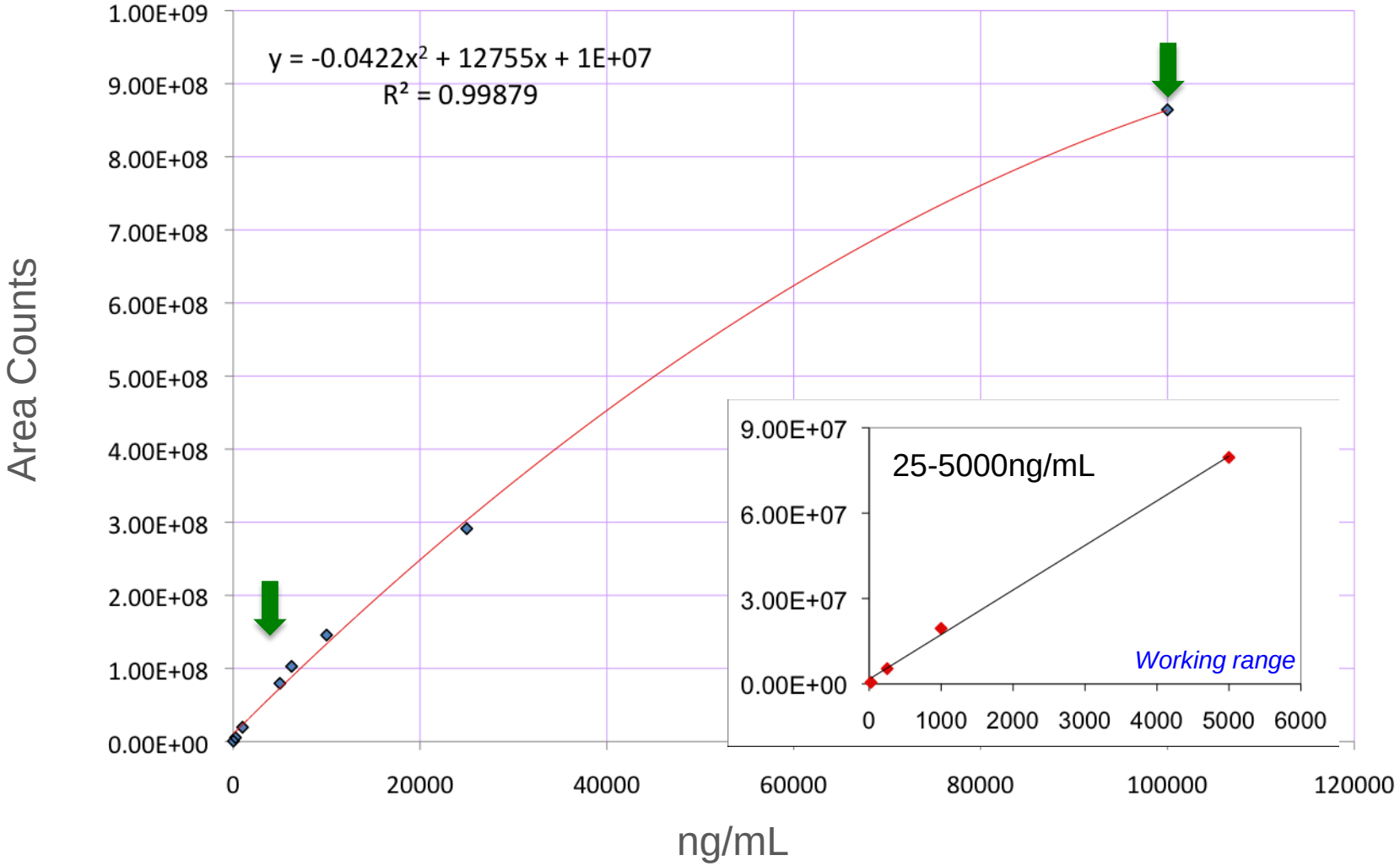
Exact Mass (specific isotope of each element)

$^{12}C_{18}{}^{1}H_{26}{}^{15}N^{16}O$ = 273.1979

In LC/MS, where we gain/lose proton H^+

H atom weighs	1.007825
H^+ weighs:	1.007276

Dynamic Range (Bioanalysis): Impact on Accurate Mass



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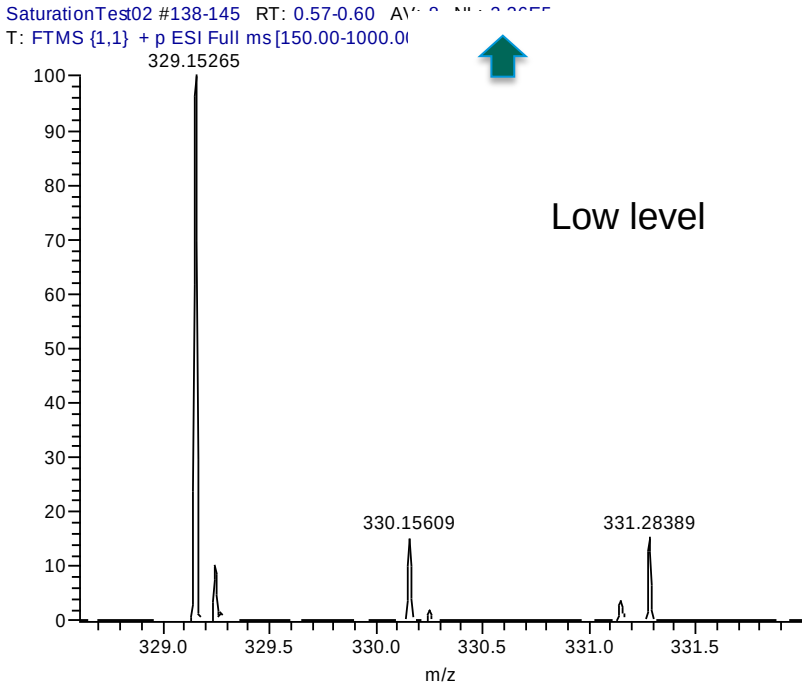
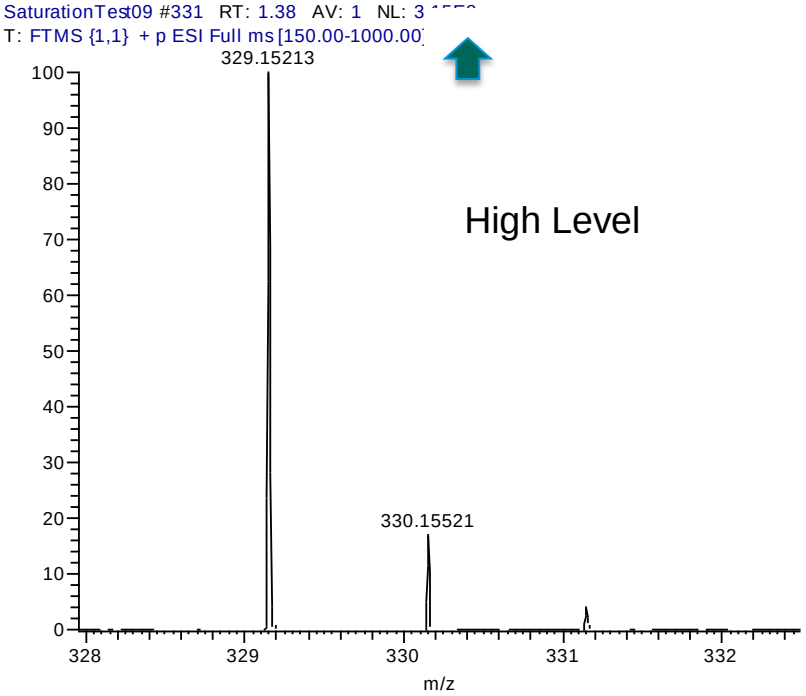
Mass Accuracy (Mass Defect Filtering) and Dynamic Range



Low level

0.9ppm mass error (25ng/mL)



High Level

2.4ppm mass error (100,000ng/mL)

Mass stability is critical – must be 3ppm or less

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DBS Case Study

FAST PK

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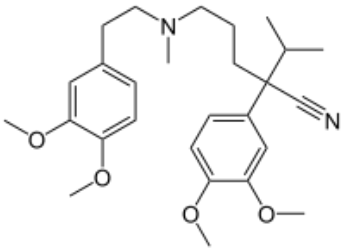
Experimental Design

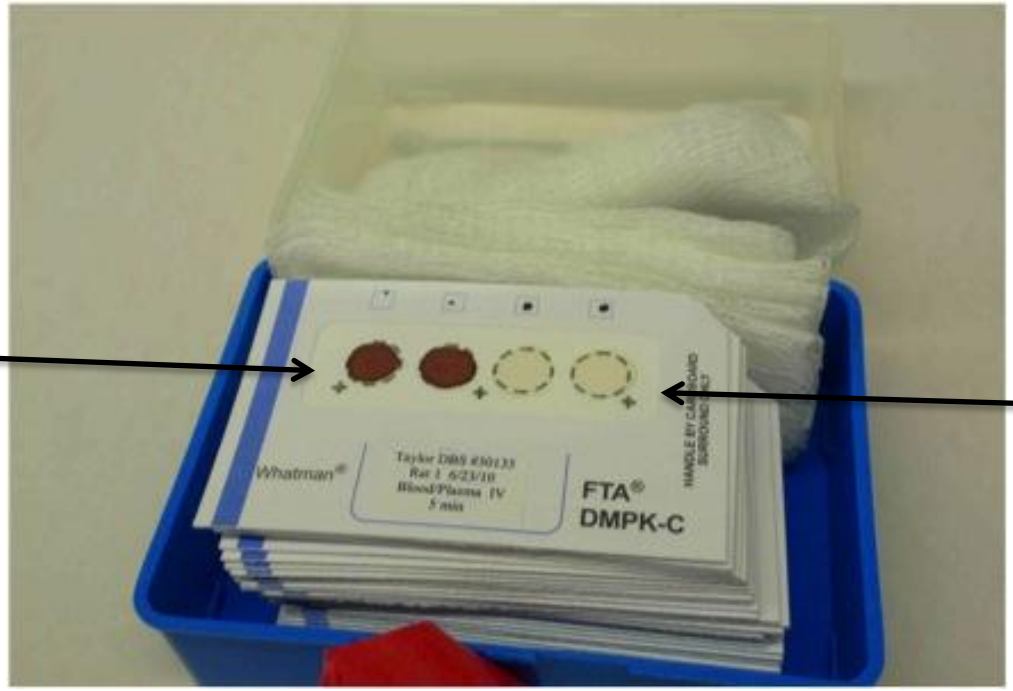


- Compound: Verapamil
- Animal model: SD Rat (with cannulae)
- Dosing: 3IV and 3PO (2-hydroxypropyl-beta-cyclodextrin)
- 6 time points
- Blood draw – spotted (DBS) and harvested for plasma at each time point
- DBS Card: FTA DMPK Uncoated (25uL spot)

- Bioanalysis
  - HRMS analysis (Full Scan High Resolution, R=25,000K, 2ppm mass accuracy)
  - Other factors:
    - > *No method developed prior to receiving samples*
    - > *No compound specific tuning performed on the Exactive (Orbitrap)*

Fast PK DBS Samples – Delivered in “Pipette” box

DBS (25uL)



D***P***S (25uL)
Can be difficult to “see” upon drying

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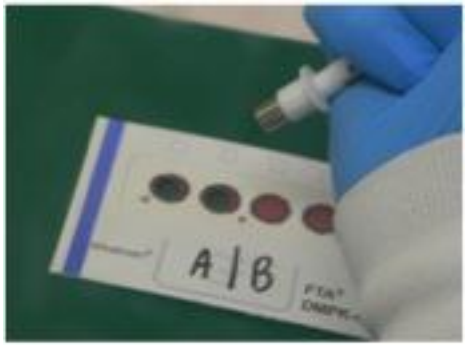
Sample Prep: Core, Transfer, Extract (in-plate), Inject



6mm core

Transfer to 96well plate

Add 100uL
MeOH-Water*
(90:10 v/v)
to each well
(contains IS)

Plate Shaker for
30 mins

Inject into
HRMS

- *No MRM Tuning*
- *Walk-up bioanalysis*
- *Full scan MS analysis (R=25K)*
- *Mass accuracy = 2ppm or less*

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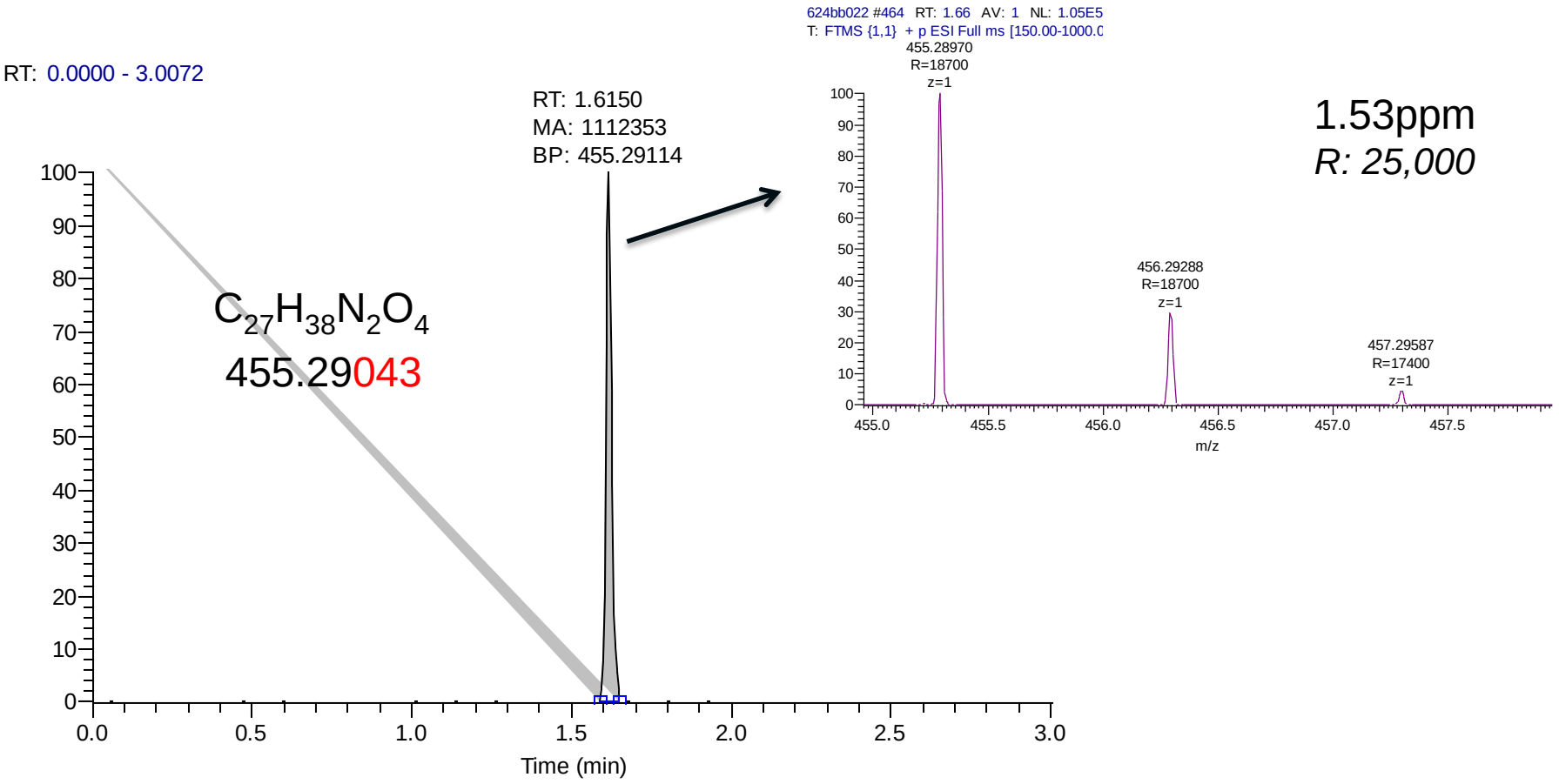
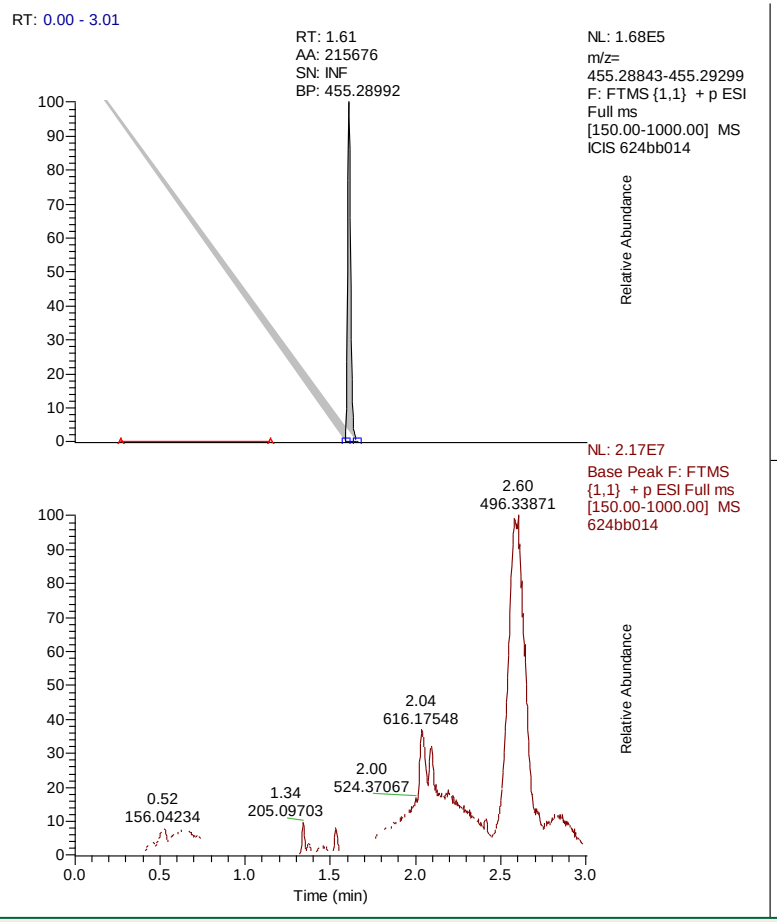
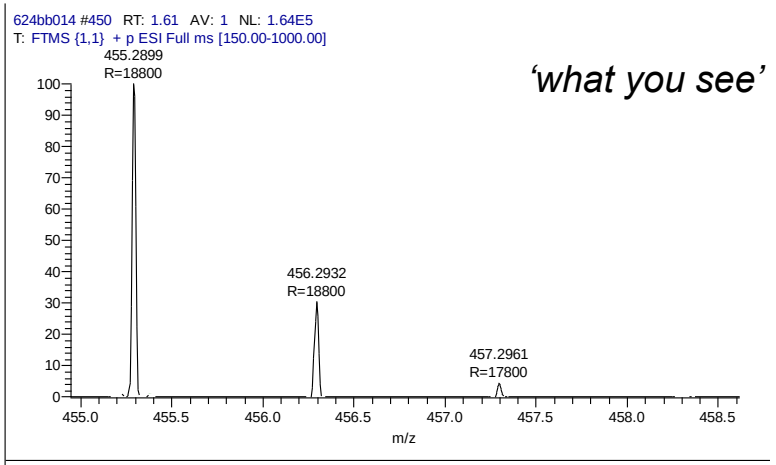
HRMS Bioanalysis of DBS for Verapamil (25ng/mL std)

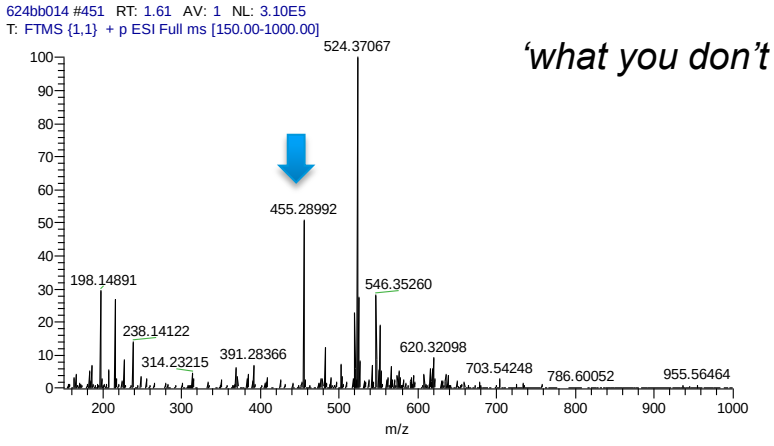


Every run can be characterized for ‘surprises’ using HRMS





‘what you see’



‘what you don’t see!’

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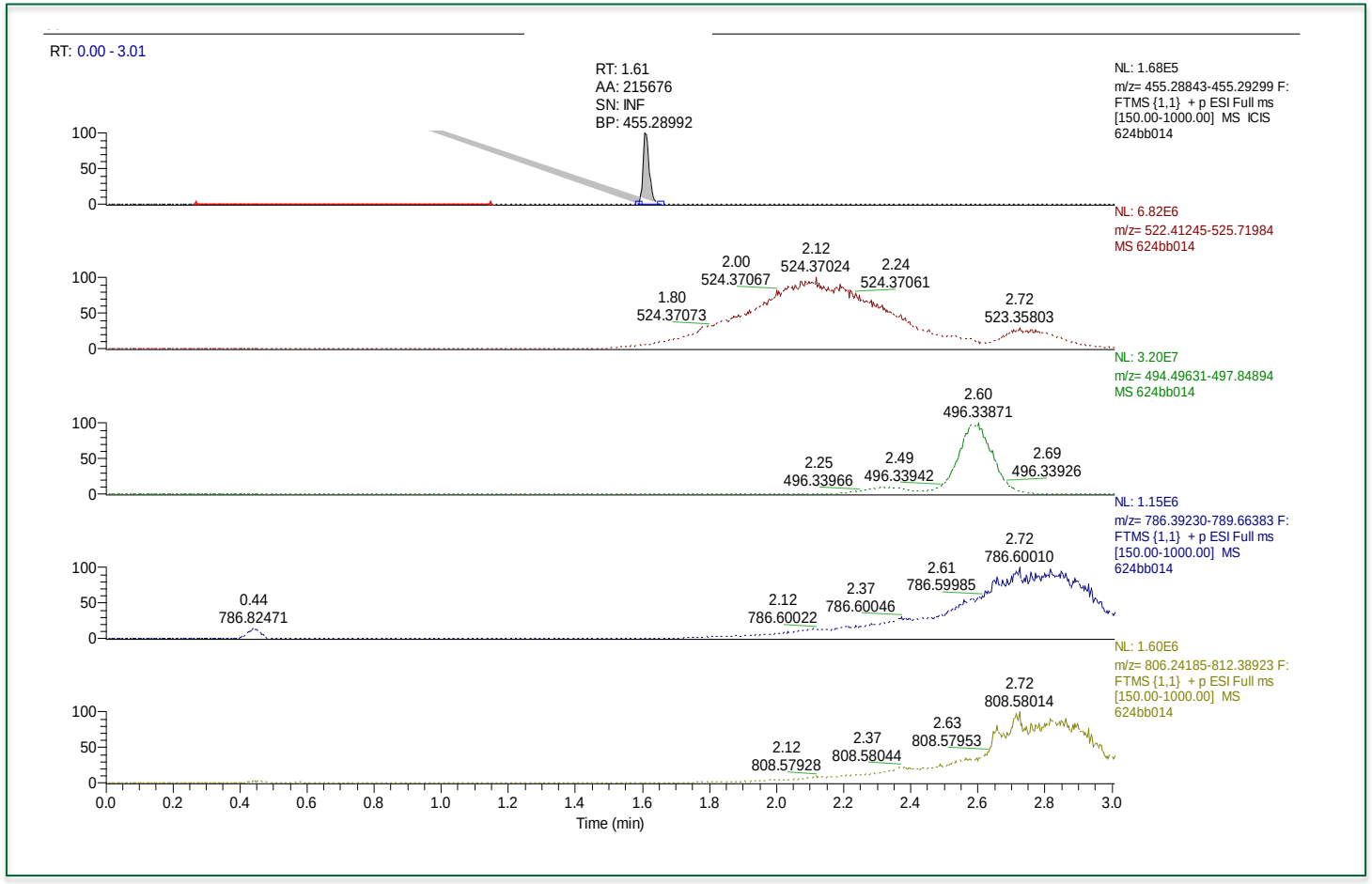
Example 1: Presence of phospholipids (ion suppression)



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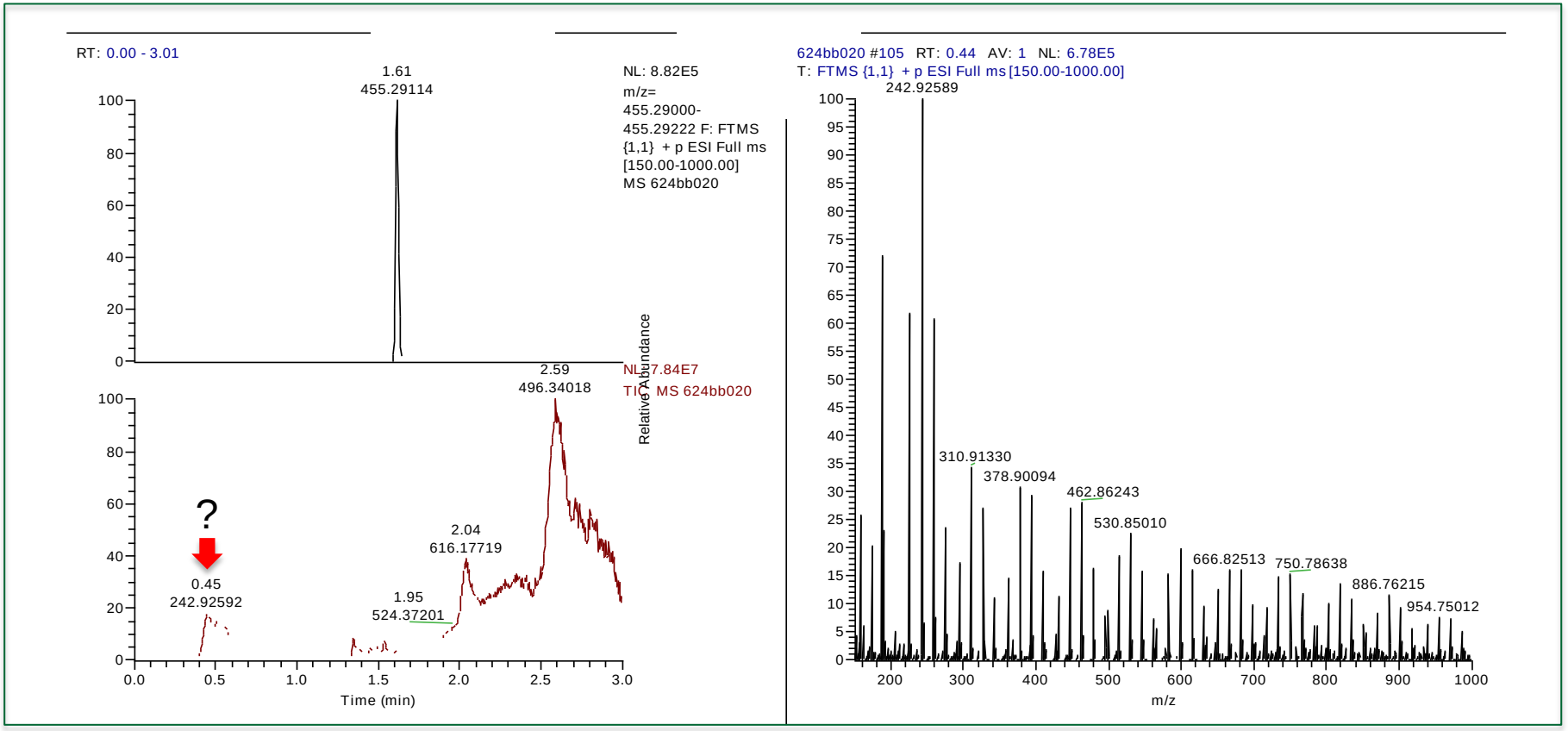
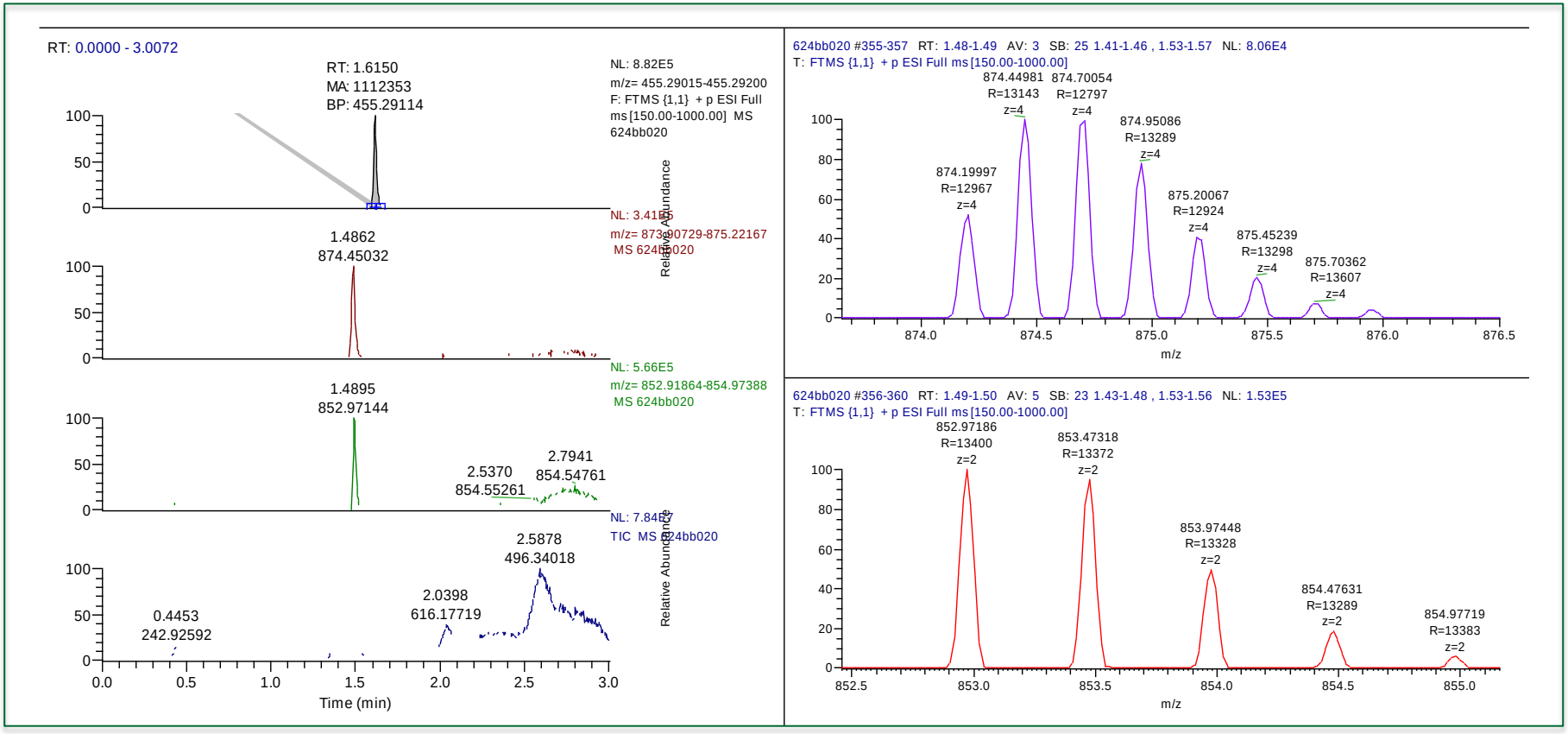
Example 2: Presence of unknown polymers/dosing vehicles



Example 3: Presence of proteins/peptides



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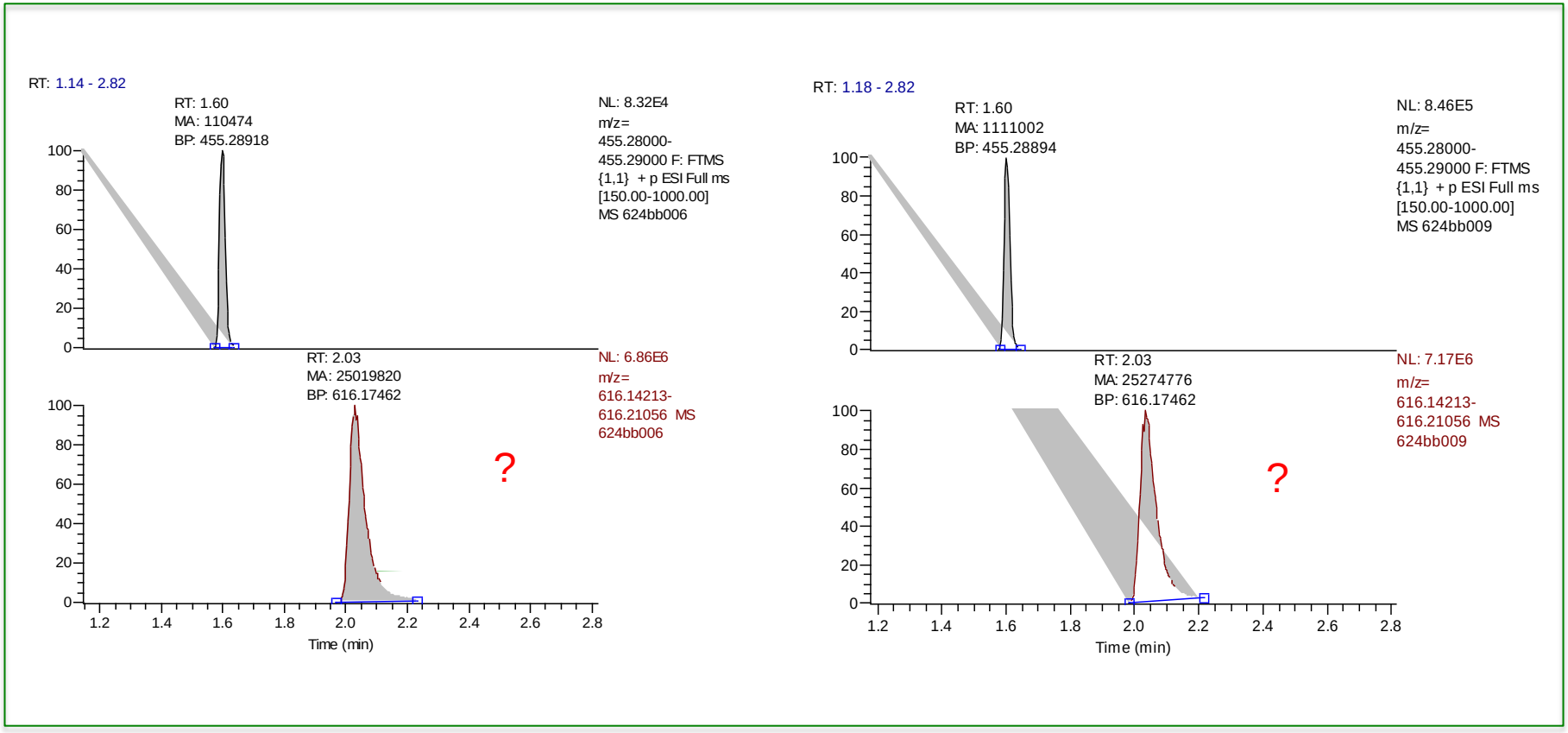
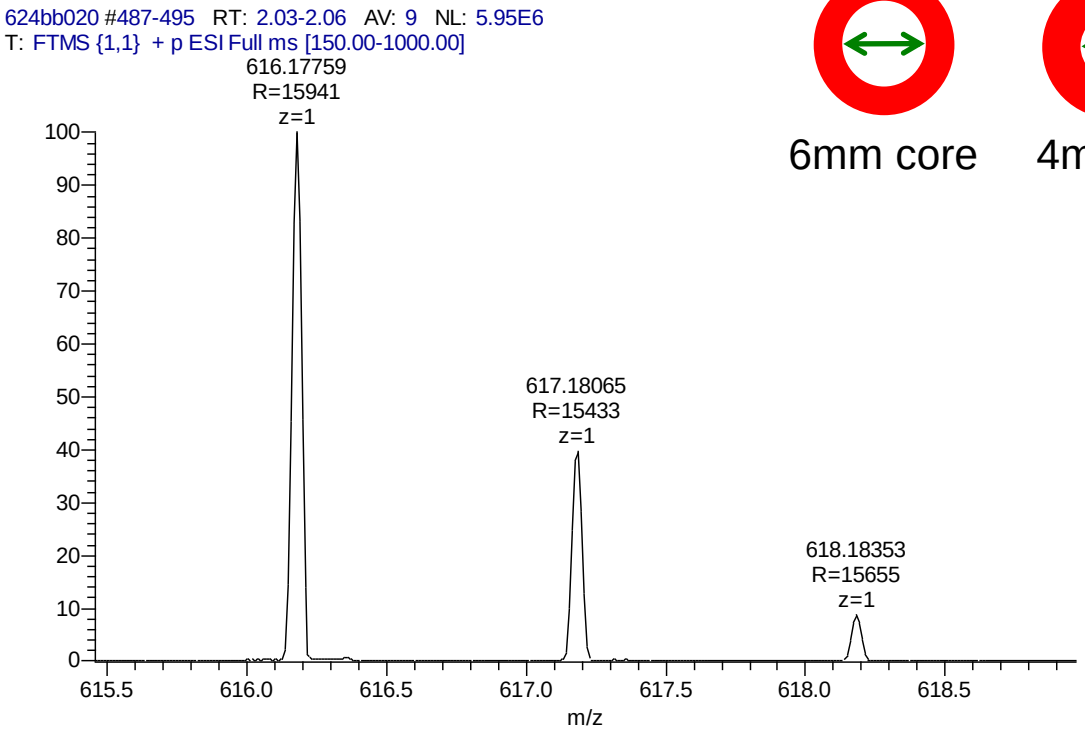
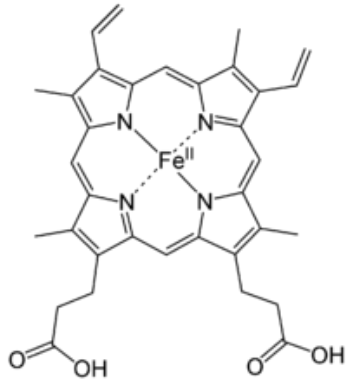
Peak Observed at ‘constant’ intensity for all DBS runs



“Heme” – Can it be used to estimate card aging, spot diameter, etc?)



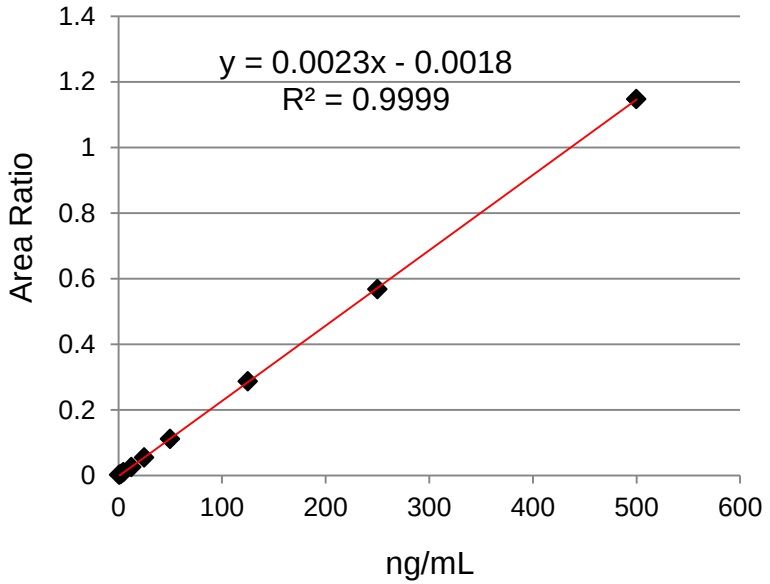


$C_{34}H_{32}O_4N_4Fe$

HRMS Regression Results (1-500ng/mL with QC's)

[Target] (ng/mL)	File Name	Peak Area Ratio	[Calculated] (ng/mL)	Standard Deviation	Precision % CV	Accuracy, % Difference
1	624bb002.q0	0.00199	1.02			2.0
1	624bb069.q0	0.00210	1.07			7.0
Mean	n=2	0.00205	1.05	0.04	3.37	5.0
2.5	624bb003.q0	0.00472	2.28			-8.8
2.5	624bb070.q0	0.00468	2.27			-9.2
Mean	n=2	0.00470	2.28	0.01	0.31	-8.8
5	624bb004.q0	0.01008	4.78			-4.4
5	624bb071.q0	0.01058	5.01			0.2
Mean	n=2	0.01033	4.90	0.16	3.32	-2.0
12.5	624bb005.q0	0.02426	11.4			-8.8
12.5	624bb072.q0	0.02731	12.8			2.4
Mean	n=2	0.02579	12.1	0.99	8.18	-3.2
25	624bb006.q0	0.05822	27.1			8.4
25	624bb073.q0	0.05268	24.5			-2.0
Mean	n=2	0.05545	25.8	1.84	7.13	3.2
50	624bb007.q0	0.11075	51.2			2.4
50	624bb074.q0	0.11293	52.2			4.4
Mean	n=2	0.11184	51.7	0.71	1.37	3.4
125	624bb008.q0	0.29358	134			7.2
125	624bb075.q0	0.28022	128			2.4
Mean	n=2	0.28690	131	4.24	3.24	4.8
250	624bb009.q0	0.56549	252			0.8
250	624bb076.q0	0.57172	255			2.0
Mean	n=2	0.56860	254	2.12	0.84	1.6
500	624bb010.q0	1.21133	516			3.2
500	624bb077.q0	1.08362	465			-7.0
Mean	n=2	1.14748	491	36.06	7.34	-1.8

[Target] (ng/mL)	File Name	Peak Area Ratio	[Calculated] (ng/mL)	Standard Deviation	Precision % CV	Accuracy, % Difference	
5	624bb015.q0	0.01014	4.81			-3.8	OK
5	624bb016.q0	0.01057	5.00			0.0	OK
5	624bb041.q0	0.01108	5.24			4.8	OK
5	624bb042.q0	0.01016	4.82			-3.6	OK
5	624bb067.q0	0.00984	4.66			-6.8	OK
Mean	n=5	0.01036	4.91	0.22	4.53	-1.8	
50	624bb014.q0	0.10640	49.2			-1.6	OK
50	624bb017.q0	0.11034	51.0			2.0	OK
50	624bb040.q0	0.10825	50.1			0.2	OK
50	624bb043.q0	0.10170	47.1			-5.8	OK
50	624bb066.q0	0.10253	47.4			-5.2	OK
Mean	n=5	0.10584	49.0	1.69	3.45	-2.0	
250	624bb013.q0	0.58211	259			3.6	OK
250	624bb018.q0	0.61337	272			8.8	OK
250	624bb039.q0	0.51941	232			-7.2	OK
250	624bb044.q0	0.56223	250			0.0	OK
250	624bb065.q0	0.58190	259			3.6	OK
Mean	n=5	0.57180	254	14.77	5.82	1.6	
Acceptance criteria (±%): 25, 25, 25							



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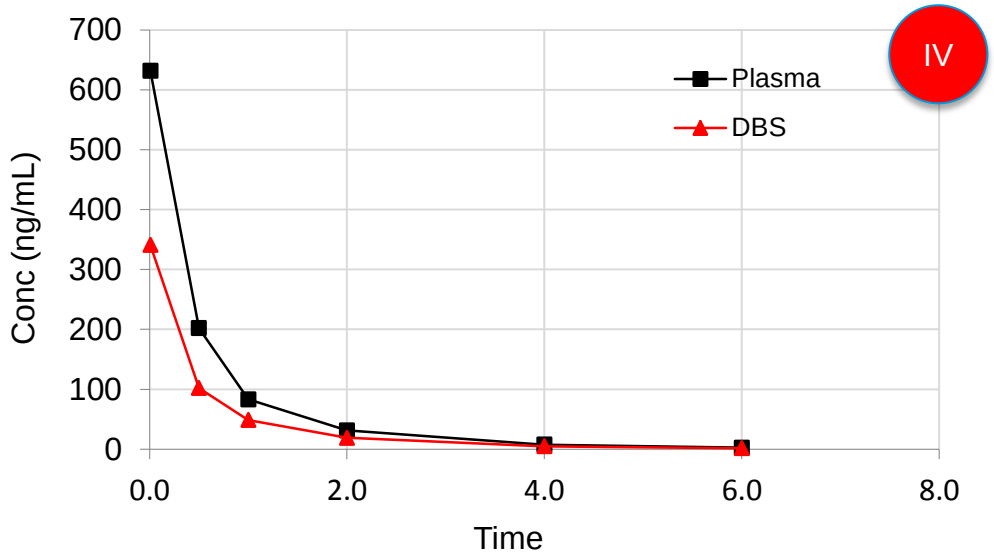
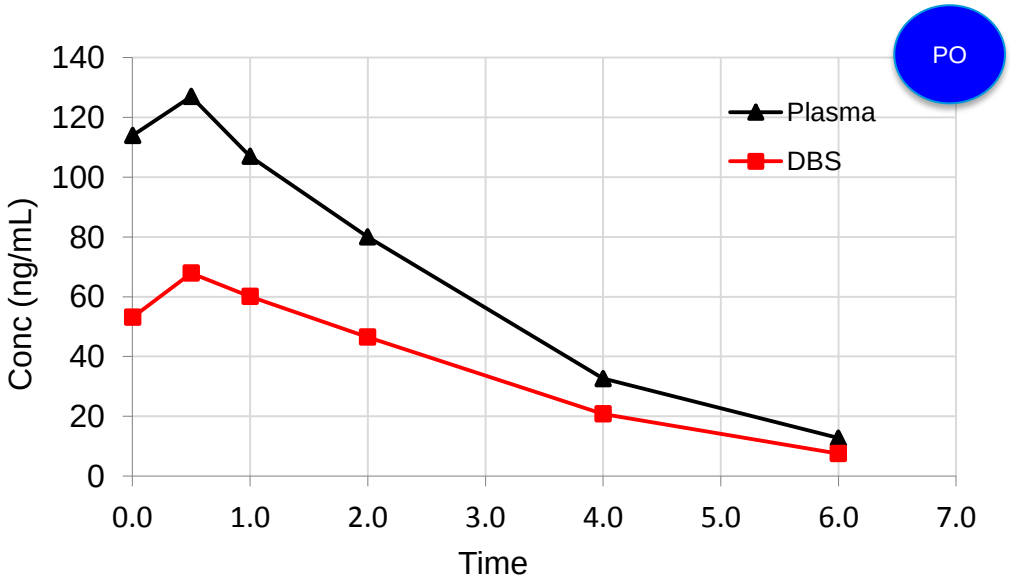
DBS v Plasma Fast PK Results





Time	Plasma	DBS	Corrected Plasma (PCV/BPR)
IV			
0.0083	632	341	353.92
0.5000	202	102	113.12
1.0000	83.1	48.5	46.54
2.0000	31.2	18.9	17.47
4.0000	7.42	4.72	4.16
6.0000	2.40	1.51	1.34
PO			
0.0042	114	53.2	66.12
0.5000	127	67.9	73.66
1.0000	107	60.1	62.06
2.0000	80.0	46.5	46.40
4.0000	32.6	20.8	18.91
6.0000	12.7	7.49	7.37

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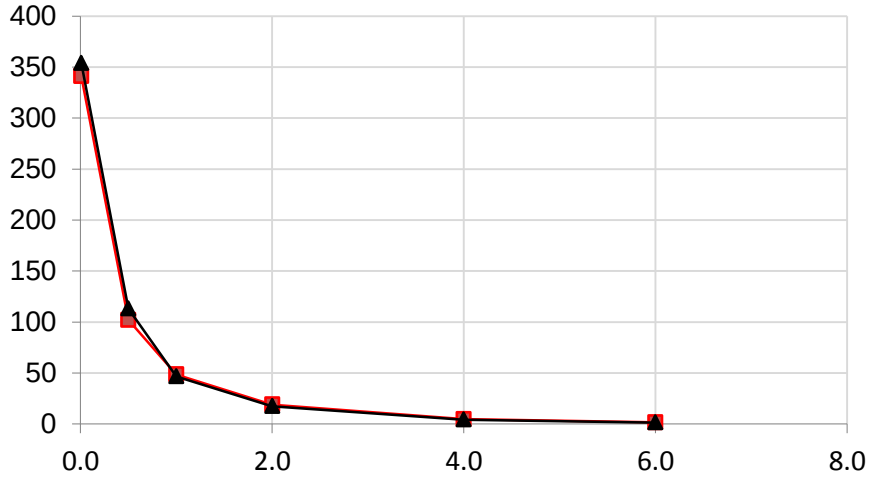
DBS vs Plasma (Bioavailability)

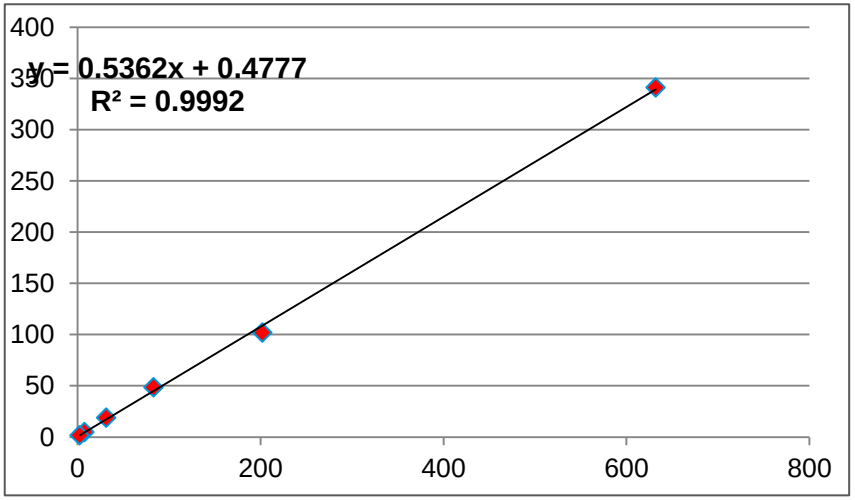
IV DBS vs Plasma Corrected

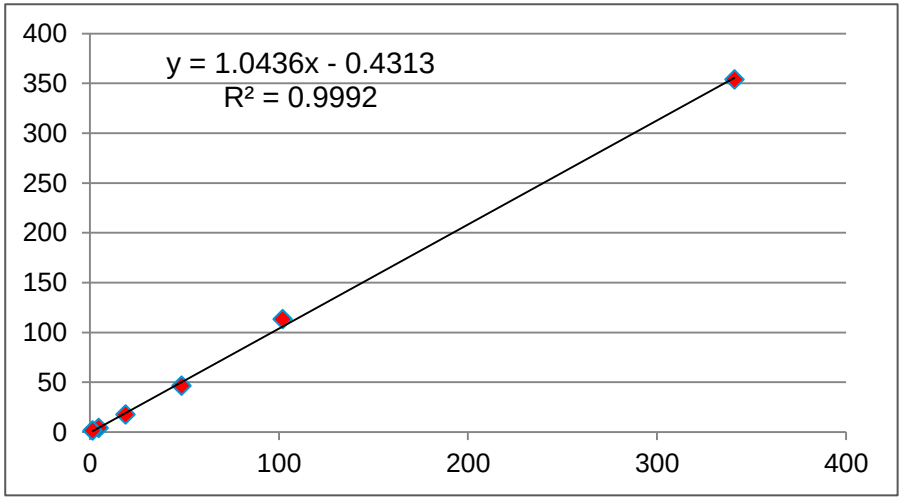


WinNon LIN Results

IV			
Parameter	**Units**	**Estimate**	**Group**
AUClast	hr*ng/mL	410.02	Plasma
AUClast	hr*ng/mL	225.75	DBS
PO			
AUClast	hr*ng/mL	354.28	Plasma
AUClast	hr*ng/mL	202.68	DBS

			Bioavailability, %
		Plasma	43.20
		DBS	44.89





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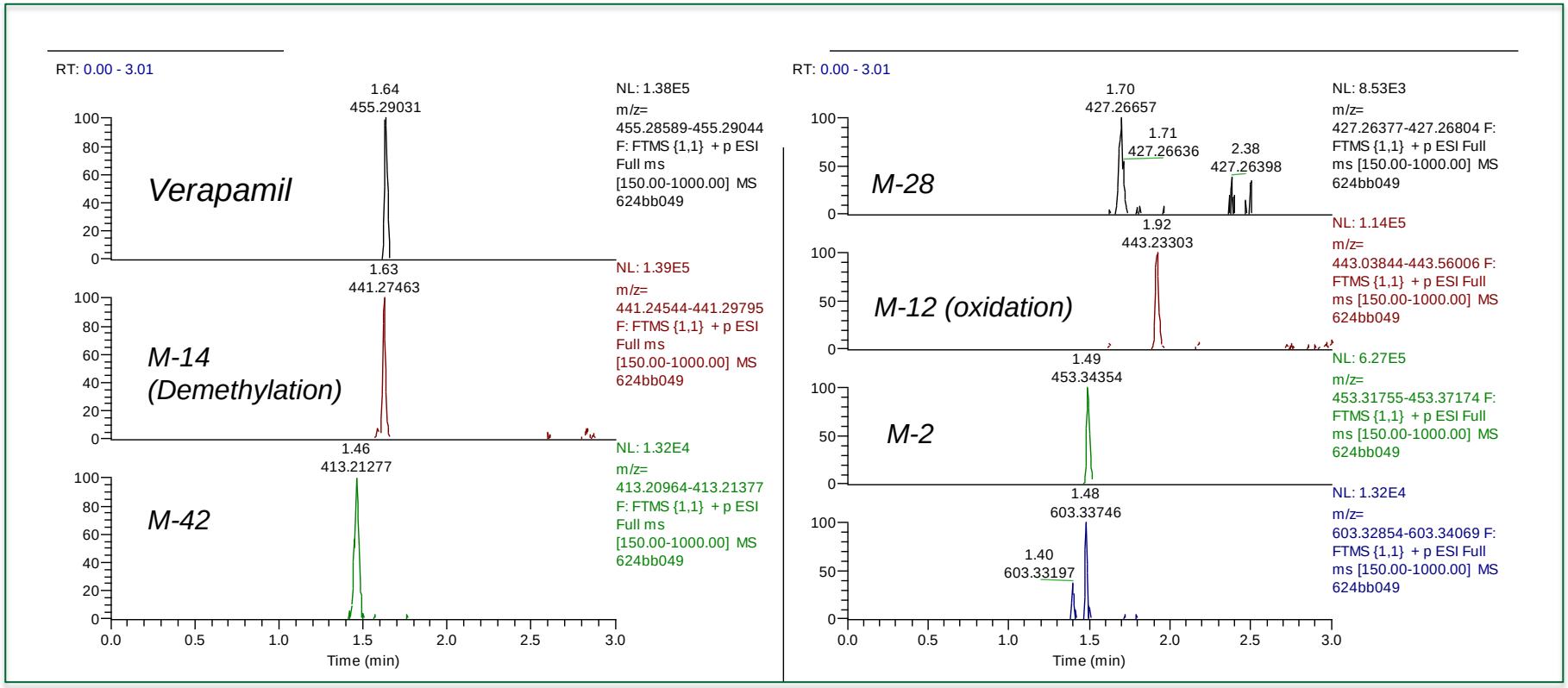
HRMS Bioanalysis: Biotransformation products & improving chemistry



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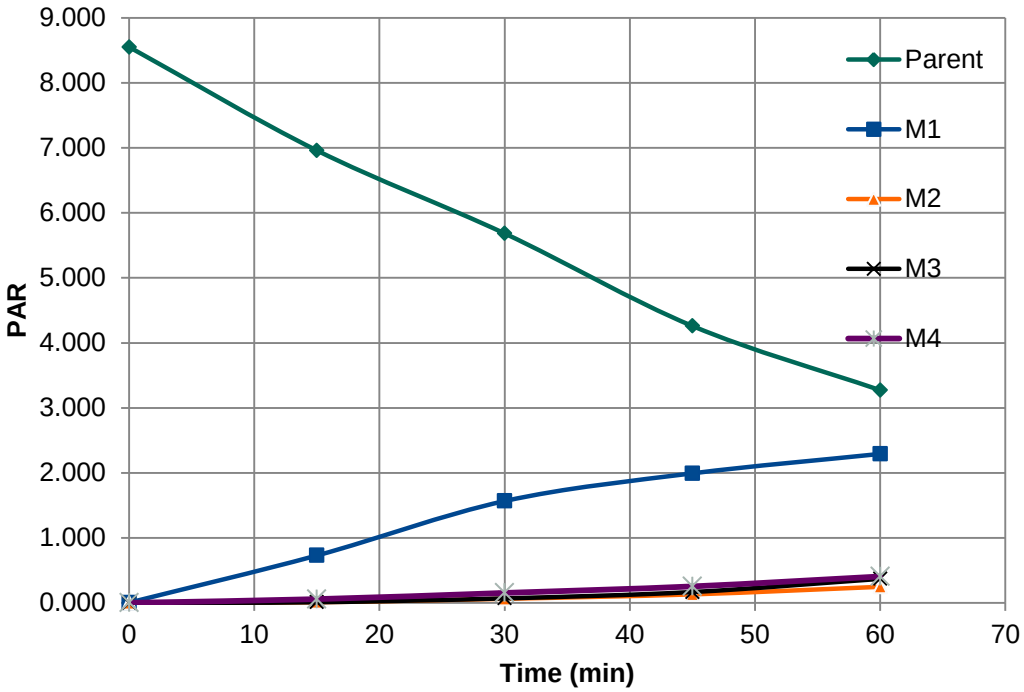
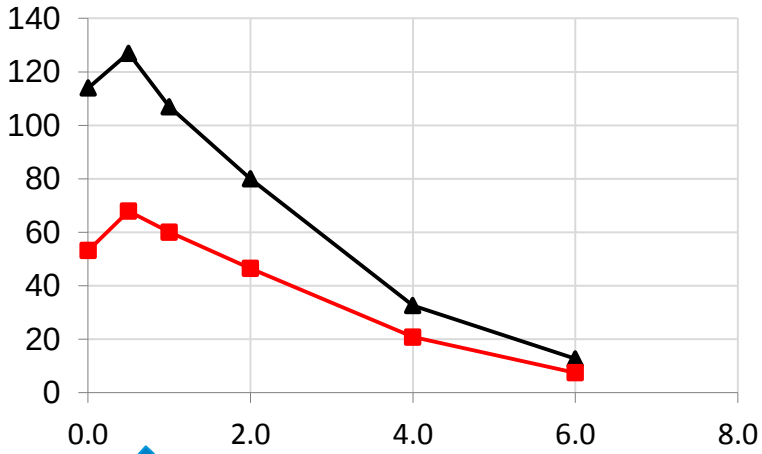
Value of HRMS Bioanalysis





Improve NCE chemistry by understanding issues with poor PK (PO)

Semi-quantitative, 'on-the-fly' analysis of Verapamil metabolites

Conclusion

- DBS and HRMS provide the potential to accelerate lead optimization by
  - Higher content ‘Information’ (metabolite) can be provided to improve candidate selection
  - NCE stability (unproven at this stage)
  - Surprises
    - > Formulation is basic & impacts bioavailability
    - > Ion suppression
- Is it time to use a new tool for discovery bioanalysis?
- DBS works, if it doesn’t, there’s always plasma!

"He may not be accurate but he's resourceful!"

Acknowledgements

- Michael Koleto
- Ben Begley
- Rich LeLacheur
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- Jamie Boulet (PSL)
- EBF organizing committee

Thank You