Fundamentals: Applications of LC/MS in small molecule drug discovery.

SUMS Seminar Series

May 21, 2020

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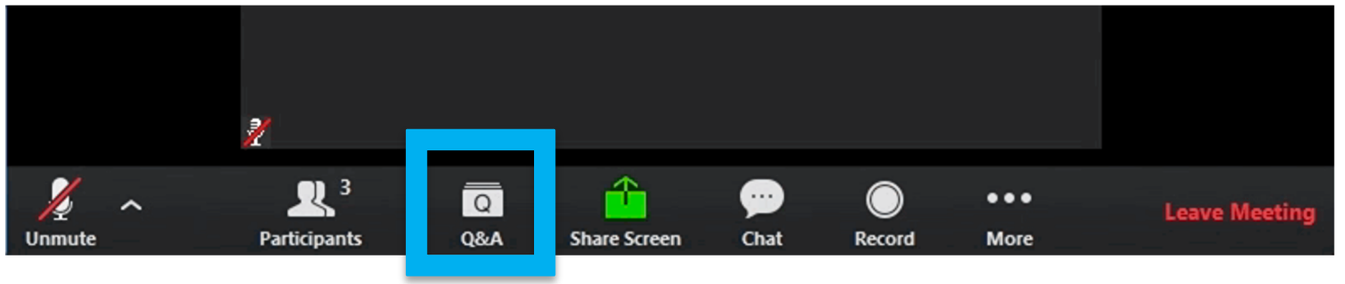
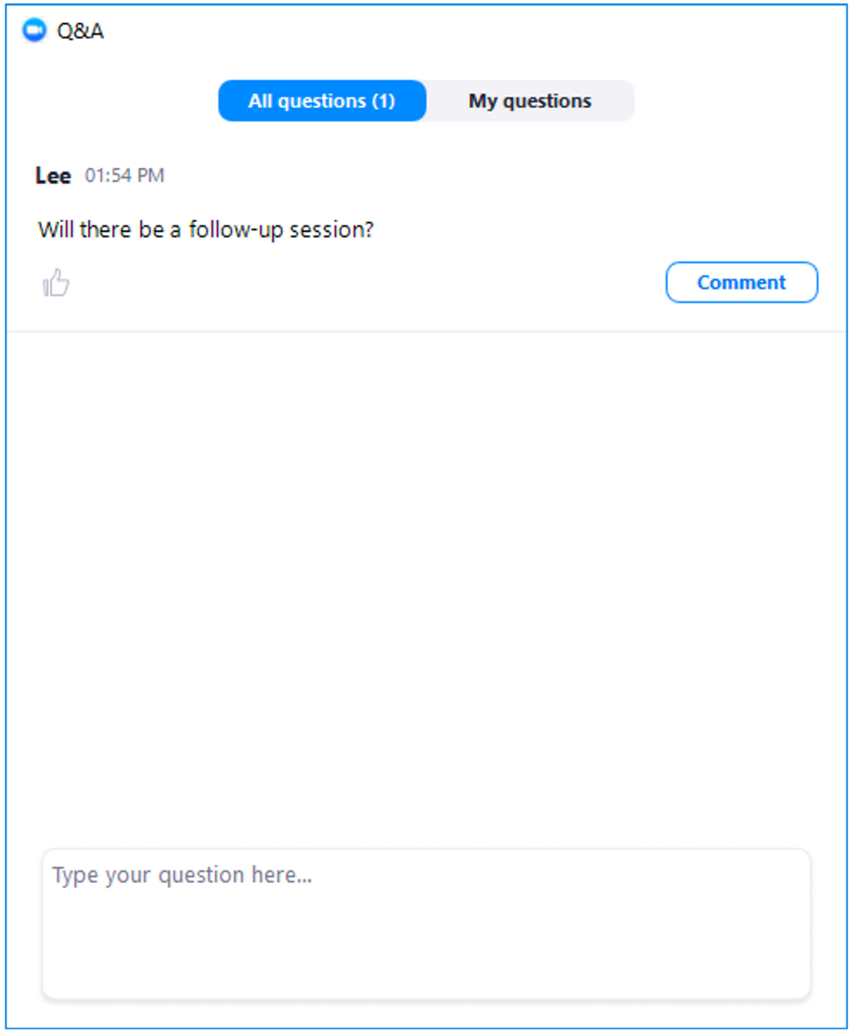
Submit your questions!





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Related Seminars in SUMS series

THURSDAY APRIL 2, 2020 - SUMS SEMINAR SERIES

Fundamentals: Measuring concentrations of small molecules using mass spectrometry - theory and practice, Part I

THURSDAY APRIL 23, 2020 - SUMS SEMINAR SERIES

Fundamentals: Measuring concentrations of small molecules using mass spectrometry - theory and practice, Part II

Recording and slides are available at https://mass-spec.stanford.edu/events

Presentation Outline

Introduction

- Drug discovery process overview
- Stages of drug discovery and development

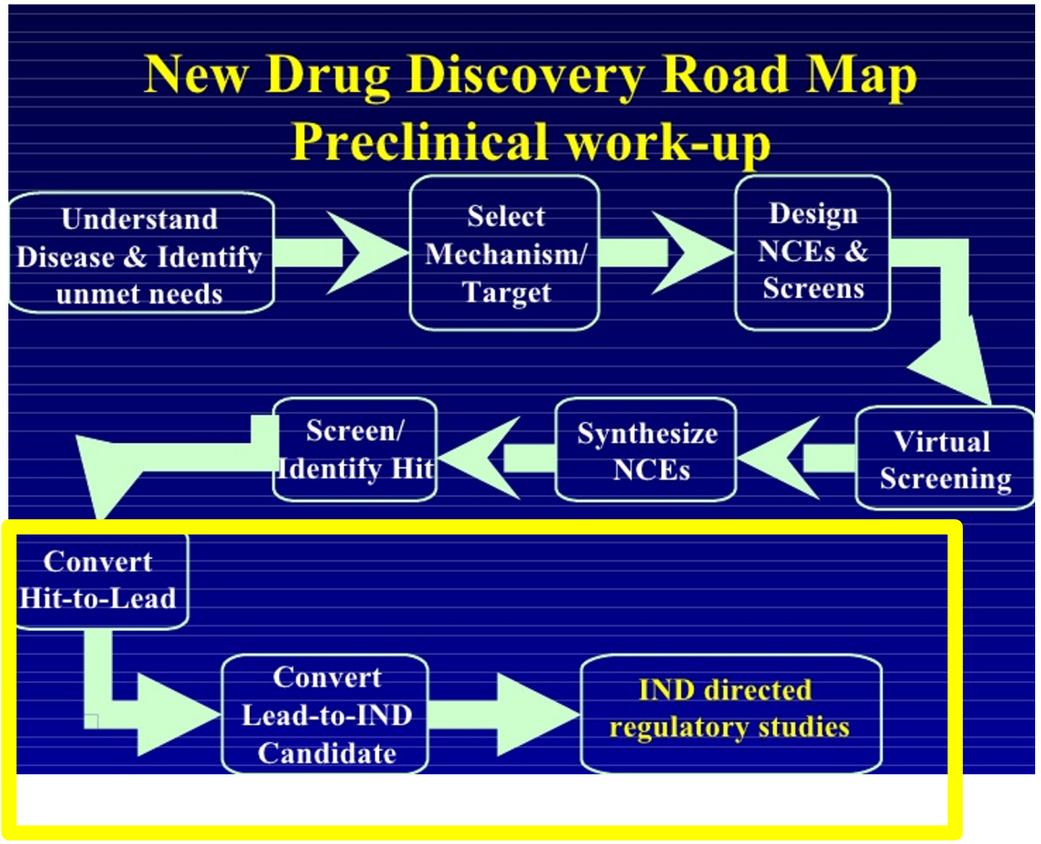
LC-MS applications in small molecule drug discovery

- Metabolism Studies
  - Pathways of drug metabolism
  - LC/MS assay requirements
  - Metabolite ID workflow
  - Metabolite ID example case study
- Pharmacokinetic (PK) Studies
  - Pharmacokinetics
  - LC-MS assay requirements
  - LC/MS PK workflow
  - PK study example

Conclusion

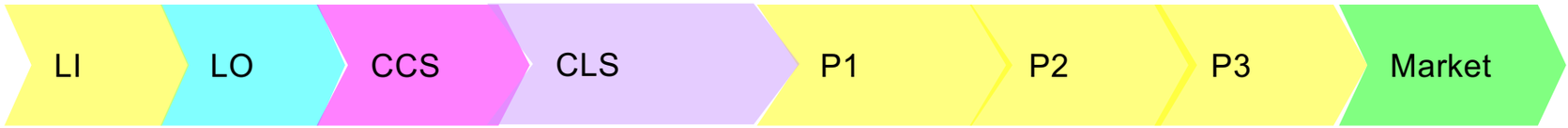
Introduction

Drug Discovery Road Map



Key goals

- Understand molecular bases of disease
- Identify a therapeutic target
- Identify new compound entities (NCEs)
- Select and optimize the best candidate
- Develop it into safe and efficacious treatment
- Regulatory submission



- Identify and profile animal metabolites
- Identify and profile human metabolites

In vitro | In vivo (cold/C^{14})

- Pharmacokinetic/ADME studies
- Metabolomics and biomarker research/Human **M**etabolites **I**n **S**afety **T**esting - MIST
- LC/MS bioanalytical methods

Parent
Metabolite

Non-GLP method | GLP method

Absorption
Distribution
Metabolism
Excretion

Metabolism, Bioanalytical and Pharmacokinetic studies are essential

LC/MS methodology is a technique of choice throughout the process

GLP: Good Laboratory Practice

Metabolism Studies

What is drug metabolism?

- Drug metabolism is a chemical modification of exogenous compounds by the body enzymes. It is the way body transforms drugs, so they can be excreted more easily.

Why do we need metabolism studies?

- Drug metabolism studies *in vitro* are conducted to determine metabolic stability and identify metabolites of parent drug to predict compound behavior in vivo preclinical models.

- Drug metabolism studies *in vivo* are conducted to define absorption, distribution, metabolism and excretion (ADME) of a drug in animal models for extrapolation to humans.

Why it is important?

- If metabolic profiles are similar in animals and humans, results from pharmacological, pharmacokinetic and toxicology studies may be more correlated with efficacy and safety in humans

- If significant difference exists, e.g. major human metabolite was not detected in animal PK/PD or toxicology studies, additional assessment of efficacy and toxicological profile of this metabolite may be needed.

Drug metabolism is normally divided into two phases:
Phase I and Phase II

Phase I Metabolism

Oxidation	Cytochrome P-450 or other enzymes (e.g. amine oxidases, dehydrogenases)
Reduction	Hepatic microsomes
Hydrolysis	Various enzymes (e.g. ester hydrolysis)
Hydration	e.g epoxide hydrolase
Other	

- Microsomes fraction (endoplasmic reticulum) of many different cells (e.g liver, kidney, lung and intestine) contain mixed-function oxidase system (Cytochrome P-450)
- It is being recognized that the main function of phase I metabolism is not to facilitate excretion, but to prepare the drug for Phase II metabolism.

Phase II Metabolism

Modification	Enzyme
Glucuronidation	UDP-glucuronyltransferase
Glycosidation	UDP-Glycosyltransferase
Sulfation	Sulfotransferase
Methylation	Methyltransferase
Acetylation	Acetyltransferase
Amino acid Conjugation	Glycine-N-acyltransferase
Glutathione conjugation	Glutathione-S-transferase
Fatty acid conjugation	Various enzymes

- Phase II reactions involve a diverse group of enzymes
- Phase II is considered to be detoxification pathway and gives rise to products which are generally more water soluble and easily excreted in bile or urine

LC/MS Assay Requirements and Challenges

Examples of Metabolism Studies

- Metabolic stability in microsomes and hepatocytes
- Metabolite identification
- Metabolite quantitation
- Pharmacokinetic (PK) studies
- ADME

Each discovery and development stage has specific goals

Discovery Stage – Identify New Drug Candidates

Identify Soft Spots in a Given Chemical Scaffold

- Metabolic stability assay in microsomes and hepatocytes
- Metabolite ID in microsomes and hepatocytes
- Identify major metabolites for unstable compounds
- Reactive metabolite screening (GSH)

- ❑ High throughput screening assays
- ❑ General LC/MS methods are utilized

Preclinical Stage - Select and Optimize the Best Candidate

Ensure that metabolites predicted to be formed in humans are being appropriately evaluated in animal PK/PD and toxicology studies

Definitive MetID assays in vitro/in vivo

- Liver Microsomes/Hepatocytes (Human, Animals)
- Plasma, Blood, Urine, Bile (Animals)

- ❑ Major and minor metabolites Identified in preclinical species
- ❑ Carefully tuned bioanalytical LC/MS methods for parent and metabolite(s) are validated

Clinical Stage - Develop the Best Candidate into Safe and Efficacious Treatment

Determine Human Metabolites

- Identify major metabolites observed in humans
- Confirm that they are present and quantified in animal toxicology studies
- Implement MIST strategy to investigate metabolite exposure

- ❑ Definitive MetID in clinical studies in plasma, urine, feces (cold)
- ❑ High-sensitivity LC/MS method developed and validated

General Analytical Approaches for Metabolism Studies

LC/Ultraviolet (UV)/Fluorescence

- Not much used due to low (if any) specificity of detectors

LC/MS

- Ion trap
- Q-TOF
- Triple quadrupole

The selection of the appropriate mass spectrometer is **critical** for successful analysis

Ionization Techniques

- APCI
  - More appropriate for poorly ionized analytes
  - Less matrix effect
- ESI
  - Mild ionization more appropriate for temperature labile molecules (e.g. phase II metabolites)

Polarity is a great tool to maximize metabolite identification!

Positive-Negative Switching

- Positive ESI is always useful in metabolite ID
- Negative ESI spectra are often useful for structure determination of drug molecules, often useful for Phase I metabolites and almost universally useful for Phase II metabolites
- Tradeoffs
- Good to collect both!

Our approach is to conduct ESI+ and ESI- separately

W. Fitch, L. He, Y. Tu, L. Alexandrova, Rapid Commun. Mass Spectrom. 2007; 21: 1661–1668

What are we using at SUMS for Metabolite ID?

- Thermo Fisher LTQ XL
- Thermo Exactive Orbitrap

- Masslynx, Metabolynx
- Analyst, Lightsight
- ACD
- Meteor
- Metasite
- Scifinder
- MetID Thermo Fisher
- Xcalibur

Ion traps are instruments of choice!

Liquid Chromatography

Role of Chromatography

- Parent and metabolites separation is important to get better fragmentation coverage for major and minor metabolites.
- To be able to see polar metabolites, longer run times required

Columns

- Selection based on analyte(s) chemistry

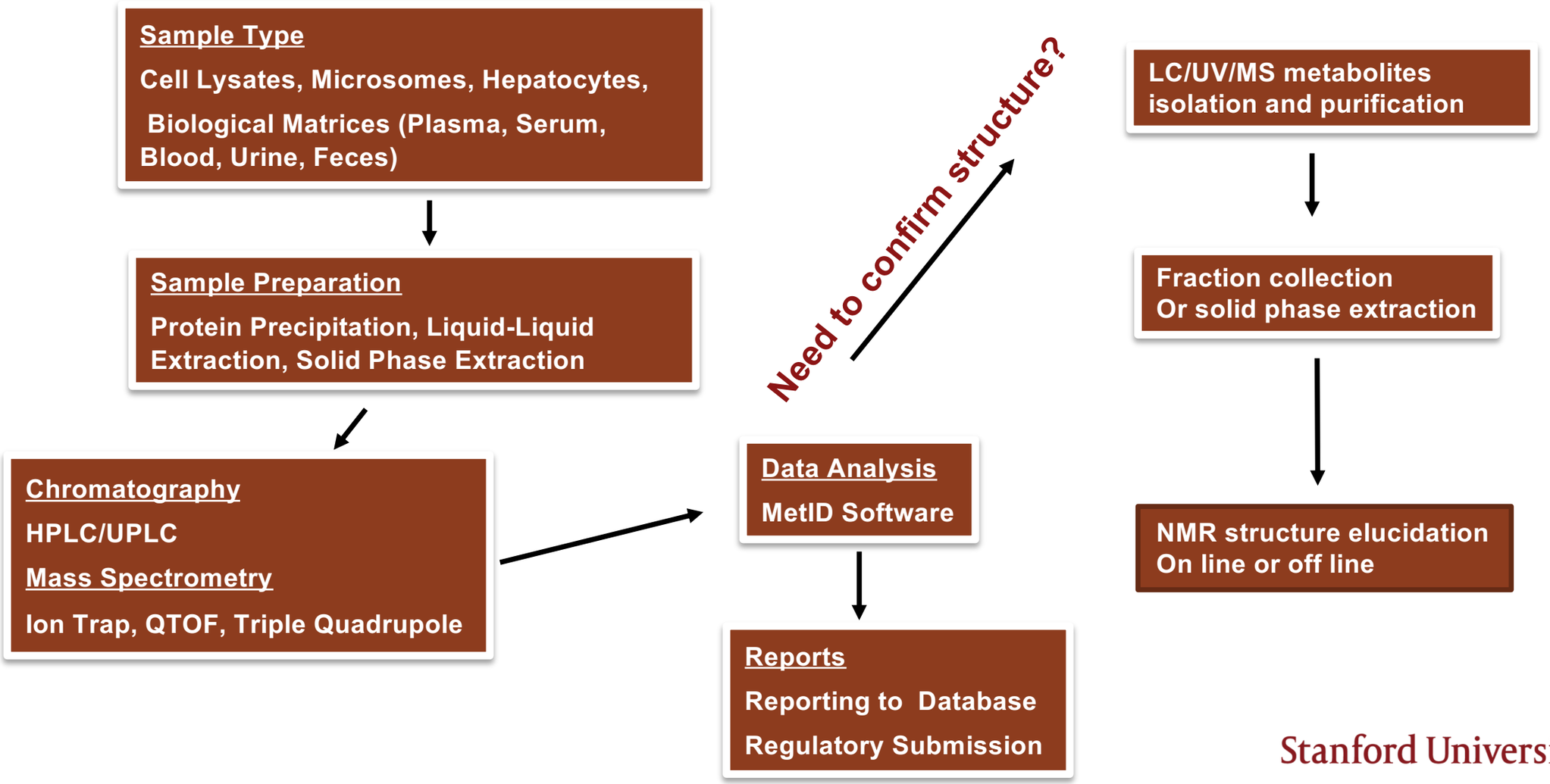
Mobile phases

- MS compatible solvents and volatile buffer modifiers
- May require adjustment for positive or negative ionization mode

Sample Preparation Techniques

- Protein Precipitation (PPT)
- Liquid-Liquid Extraction (LLE)
- Solid Phase Extraction (SPE)
  - On line
  - Off line

Metabolism Studies Workflow



Metabolite ID Example Project

Methamphetamine metabolism by pulmonary microvascular endothelial cells (PMVECs)

Mark E. Orcholski, Artyom Khurshudyan, Elya A. Shamskhou, Ke Yuan, Ian Y. Chen,
Sean D. Kodani, Christophe Morisseau, Bruce D. Hammock, Ellen M. Hong, Ludmila Alexandrova,
Tero-Pekka Alastalo, Gerald Berry, Roham T. Zamanian, and Vinicio A. de Jesus Perez. Am J Physiol Lung Cell Mol Physiol 313: L252–L266, 2017.

Metabolite ID Case Study

- Pulmonary arterial hypertension is a complication of methamphetamine use (METH-PAH)
- LC/MS metabolism study of Methamphetamine (METH) was conducted in healthy pulmonary microvascular endothelial cells (PMVECs)
- PMVECs were treated with METH for 4 hours

LC/MS Conditions

Thermo LTQ XL ion trap mass spectrometer

HESI+/HESI-

Full scan MS (110-600Da);

DDA; Dynamic exclusion

Survey scans in positive and negative modes were conducted to establish metabolite profile

Full scan and full scan MS^2 m/z 166.2 @CID 35.00 (hydroxylated methamphetamine metabolites) for structure identification

LC conditions

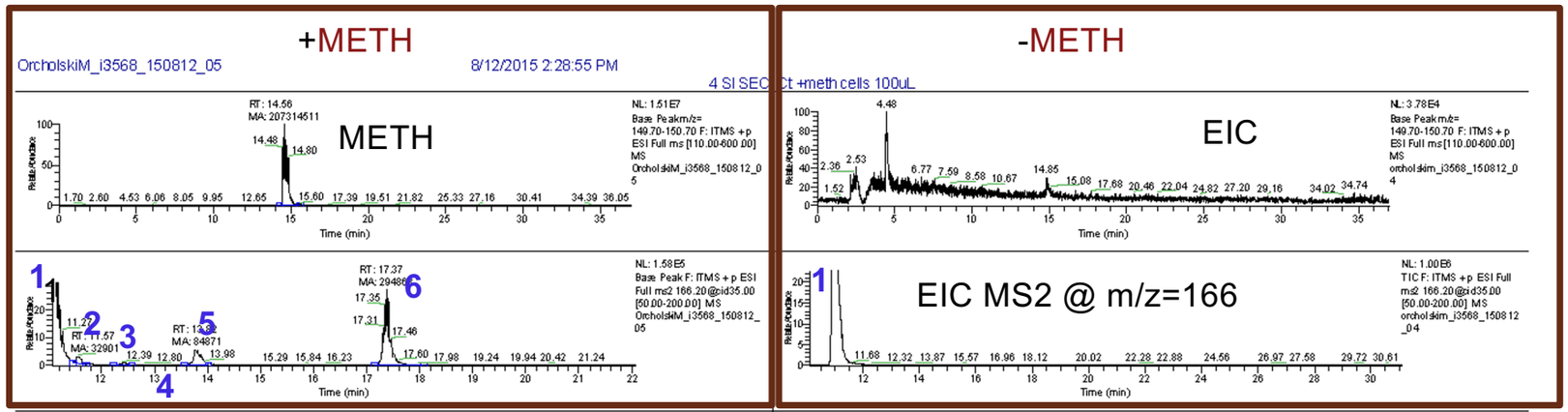
- Column: Varian Polaris; 250x2.1mm; C18A 5um
- Flow rate: 0.250mL/min
- Mobile Phase:
  - A - 0.1% formic acid water
  - B- 0.1% formic acid acetonitrile
- Gradient: 0 – 95% B in 32 minutes

Sample preparation

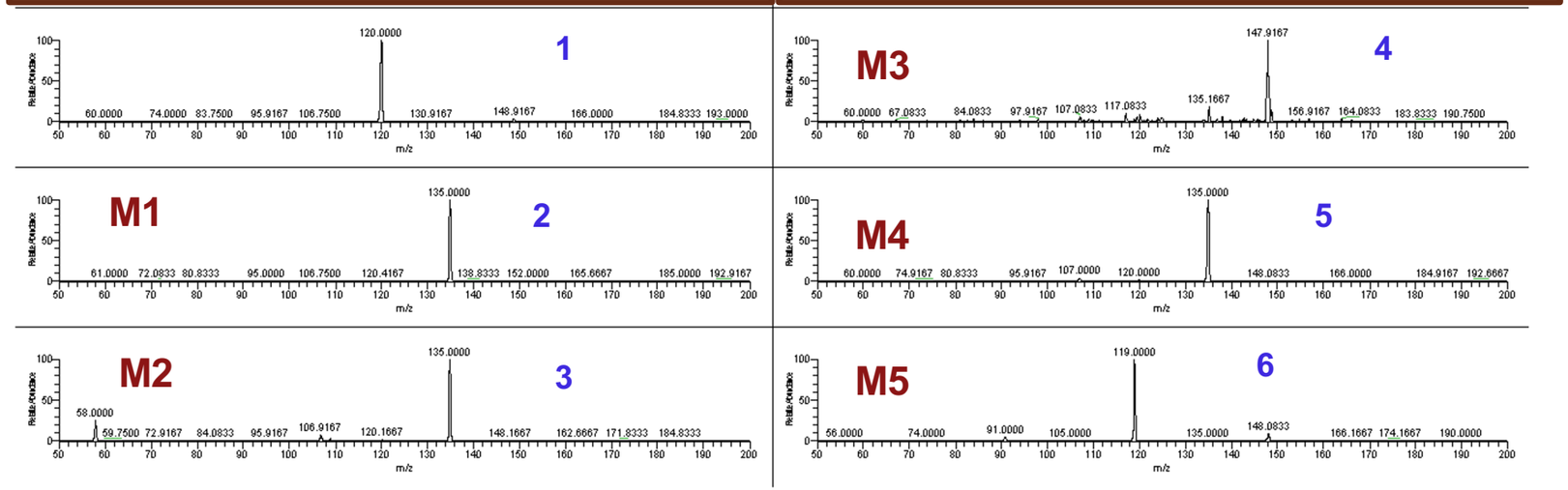
- 100uL cell suspension
- ice cold methanol
- collect supernatant
- evaporate
- reconstitute in 50uL water

PMVECs Extracts

MS Chromatograms



MS/MS Spectra



Peak 1: endogenous; Peak 2, 3, 4, 5, 6: Hydroxy methamphetamine metabolites

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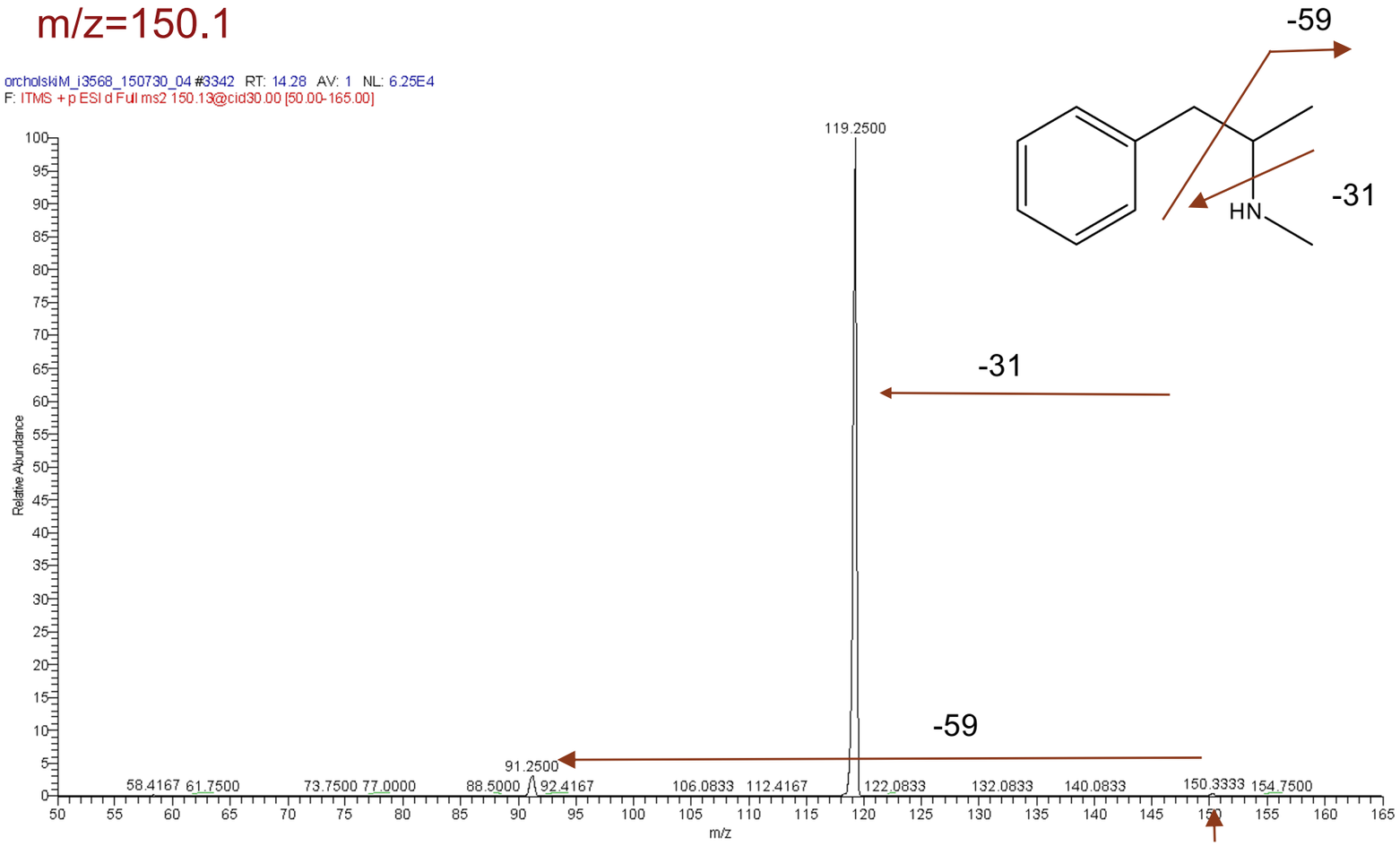
Methamphetamine MS/MS data

m/z=150.1

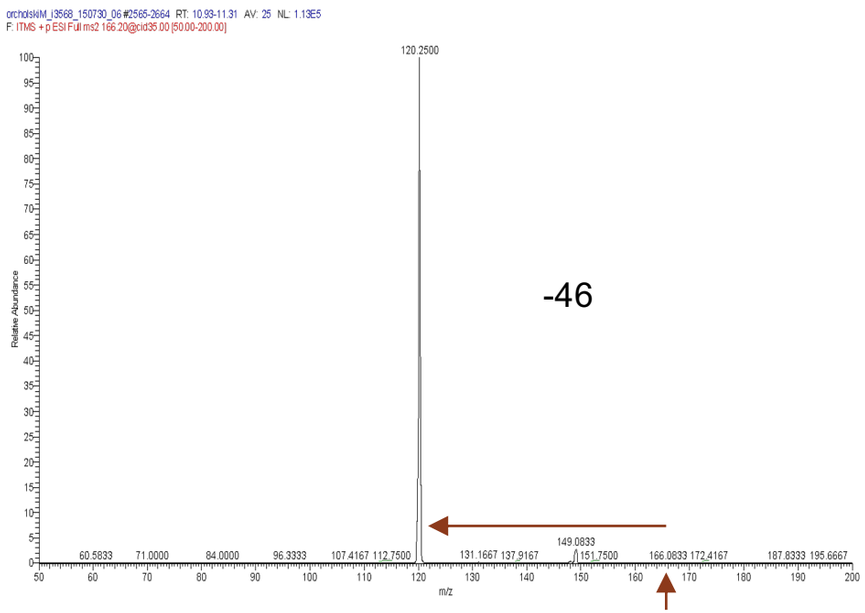


Always start from the Parent!

MS/MS Data for Metabolites

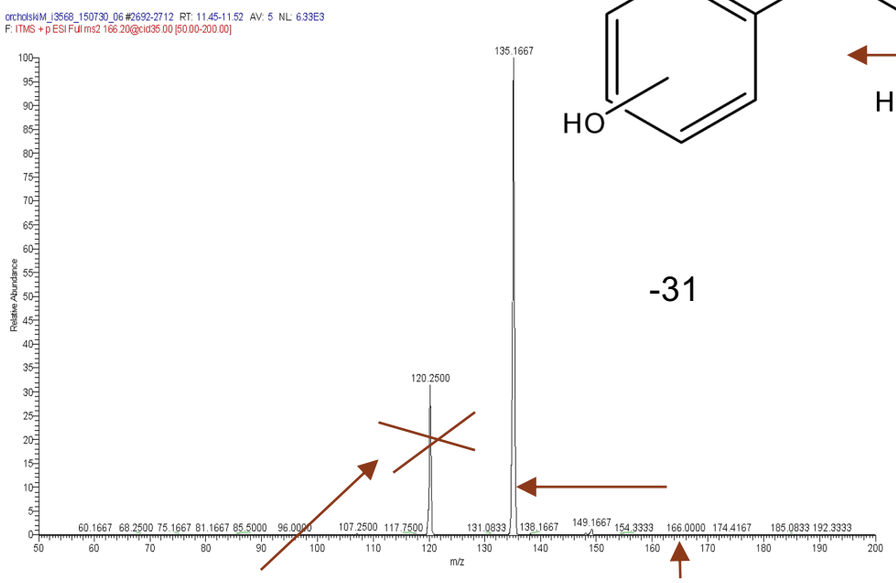
Peak 1: endogenous interference

m/z=166.2



Peak 2=M1: hydroxy-methamphetamine

m/z=166.2

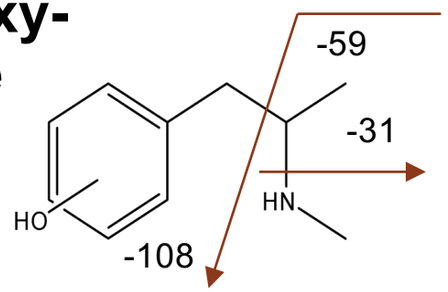


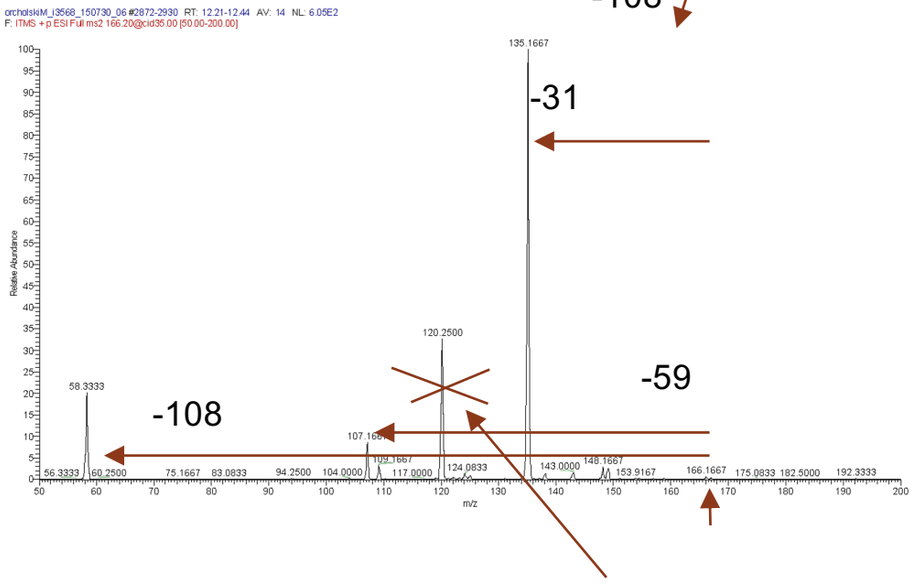
Interference from endogenous

MS/MS Data for Metabolites

Peak 3=M2: hydroxy-methamphetamine

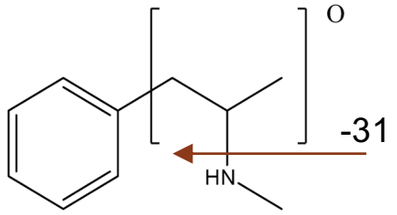
m/z=166.2

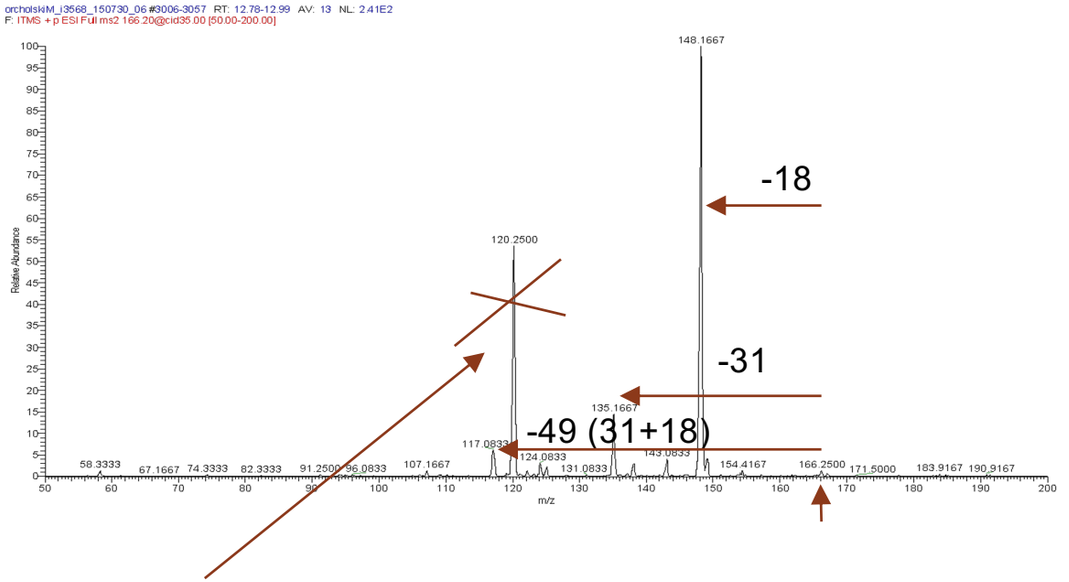




Peak 4=M3: hydroxy-methamphetamine

m/z=166.2



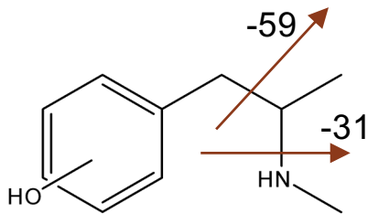


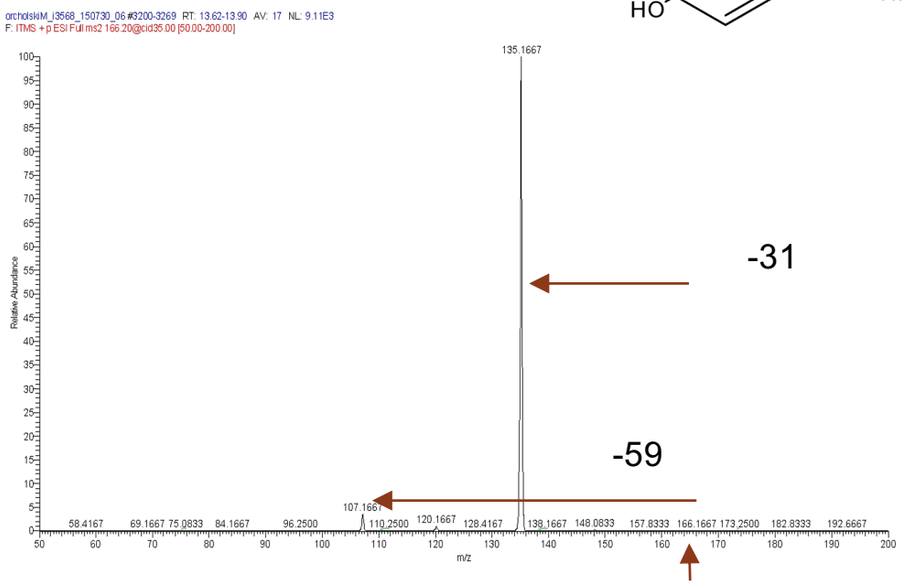
Interference from endogenous

MS/MS Data for Metabolites

Peak 5=M4: hydroxy-methamphetamine

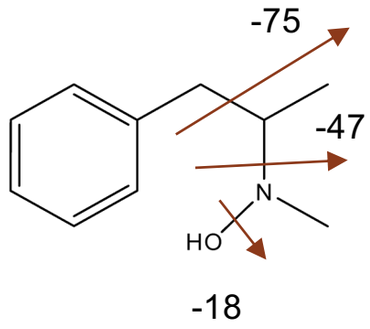
m/z=166.2

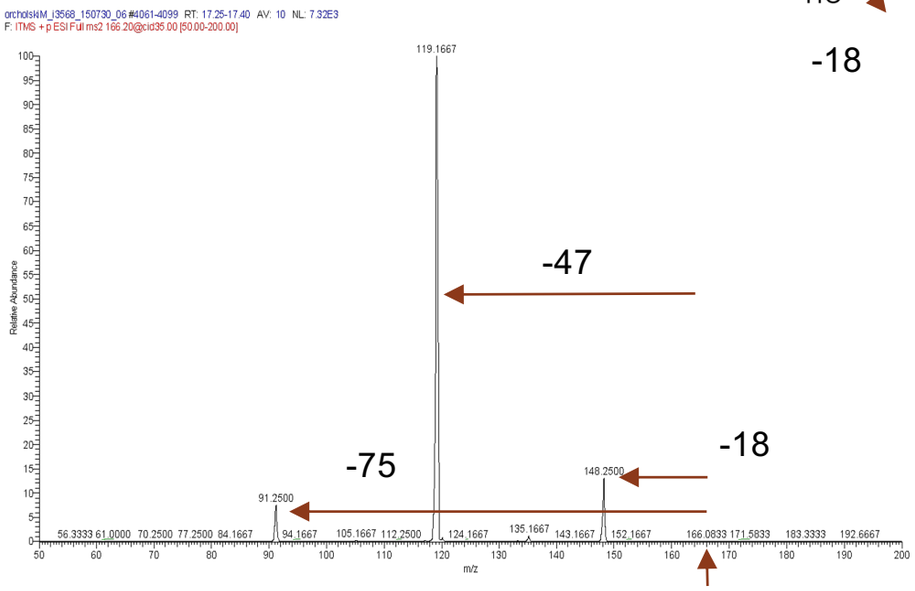




Peak 6=M5: hydroxy-methamphetamine

m/z=166.2





Possibly N-Hydroxy Methamphetamine (eluting after parent)

Results

- PMVECs have the capacity to both internalize and metabolize METH
- Peak 1 is a nonspecific endogenous component also found in unstimulated PMVECs
- Analysis of cell lysates demonstrated at least 5 hydroxy-methamphetamine metabolites (M1 – M5)
- M5, M4 and M1 are major metabolites (based on ionization efficiency)
- No phase II metabolites observed

Mark E. Orcholski, Artyom Khurshudyan, Elya A. Shamskhou, Ke Yuan, Ian Y. Chen,
Sean D. Kodani, Christophe Morisseau, Bruce D. Hammock, Ellen M. Hong, Ludmila Alexandrova,
Tero-Pekka Alastalo, Gerald Berry, Roham T. Zamanian, and Vinicio A. de Jesus Perez. Am J Physiol Lung Cell Mol Physiol 313: L252–L266, 2017.

LC/MS Applications for Pharmacokinetic Studies (PK) in Small Molecule Drug Discovery

What is Pharmacokinetics

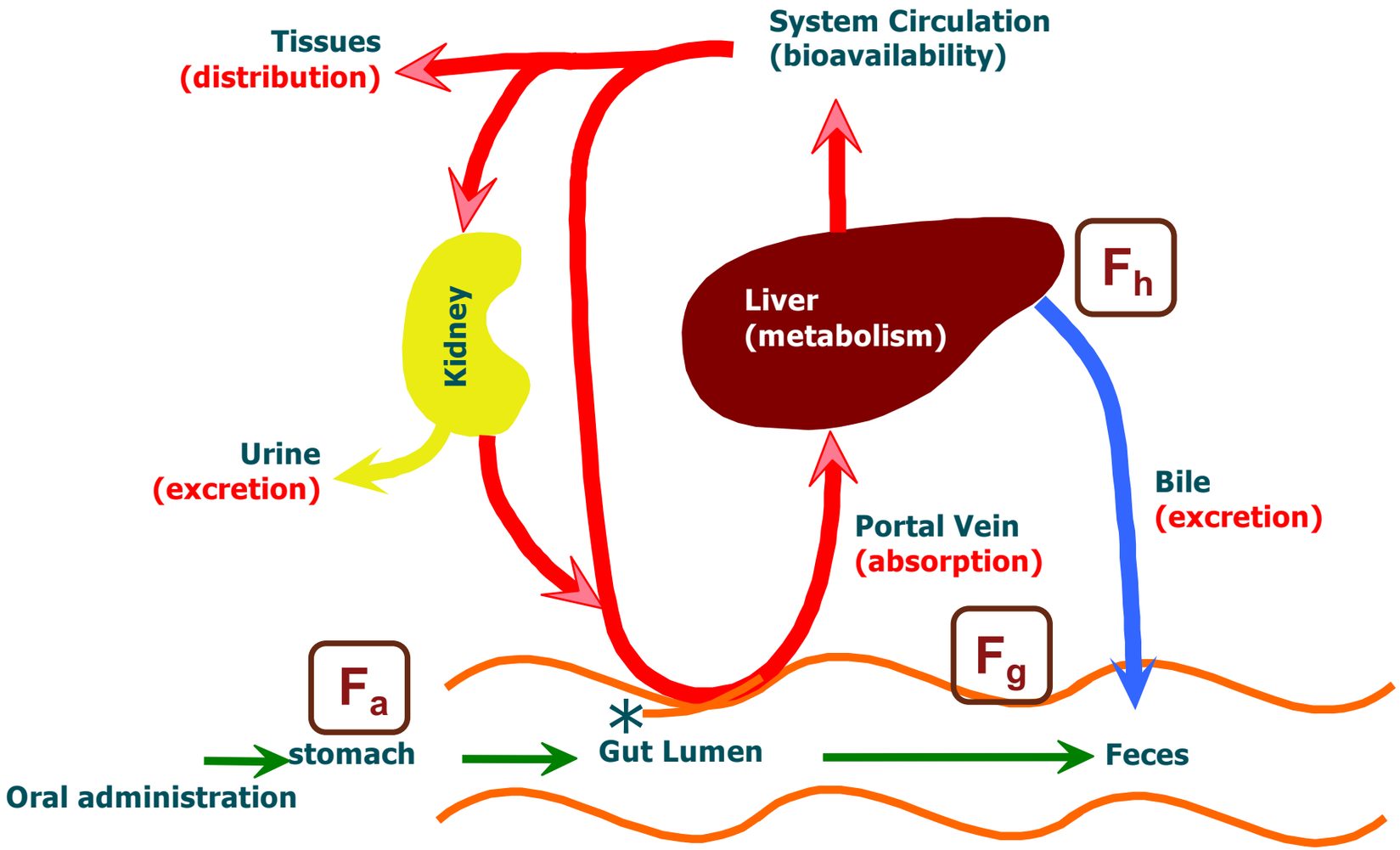
- PK study investigates how body effects a drug from time of administration to the time of complete elimination.
- Pharmacodynamic study (PD) investigates how drug effects the body.
- PK studies in animals are routine components of drug discovery workflow

Primary value of PK studies in animals is to validate in vitro and in silico approach which is used to predict pharmacokinetics in man

- Sites of administration: intravascular and extravascular
  - Intravascular: intravenously or intra-arterially
  - Extravascular: oral, sublingual, buccal, intramuscular, subcutaneous, dermal, pulmonary and rectal routes

- Measuring a drug in the body is usually in blood or plasma
  - Practical
  - Convenient
  - Carries to all organs

ADME – Pharmacokinetics – Plasma Drug Concentrations



Absorption
Distribution
Metabolism
Excretion

Bioavailability F (%)=F_a*F_g*F_h

LC/MS PK Workflow

PK Sample Analysis Workflow

- PK Study: PK study design
- Reference Standards: Analyte standard and Internal Standard
- Mass spectrometry: Automated MS tuning
- Chromatography: column/mobile phase selection; Gradient acquisition time 5-10min
- LC/MS/MS method set up in the instrument software
- Sample Extraction: Protein precipitation
- Sample acquisition: Vendor specific software
- Data analysis: vendor specific software/Excel-PowerPoint report

PK Study

- Animal dosing protocol
- Prepare dose solution
- Dose animals
- Collect plasma

For PK studies: Triple Quadrupole is Instrument of Choice

Quantitative Assays are Performed in Selected Reaction Monitoring (SRM) Mode

- Sensitivity
- Selectivity and Specificity

Waters Xevo TQ-XS
NIH grant S10OD026962

SUMS seminar series

Fundamentals: Measuring concentrations of small molecules using mass spectrometry - theory and practice, Part I

Fundamentals: Measuring concentrations of small molecules using mass spectrometry - theory and practice, part II

Mass Spectrometry and Chromatography

Reference Standards

- **Prepare analyte Stock solution**
- **Prepare Internal Standard stock solution**

MS Tuning

- **Acquire MS and MS/MS spectra**
- Optimize precursor ion – fragment ion MS parameters
- SRM transitions entered in instrument software

Chromatography

- **Column: reversed phase chromatography preferred**
- Mobile phase selection
- **Gradient elution**
- LC conditions entered in instrument software

Sample Preparation

Calibration curve

- Prepare calibration spiking solutions
- Spike standards into plasma for calibration curve
- Spike Internal standards

Samples

- Aliquot unknowns
- Spike Internal standard

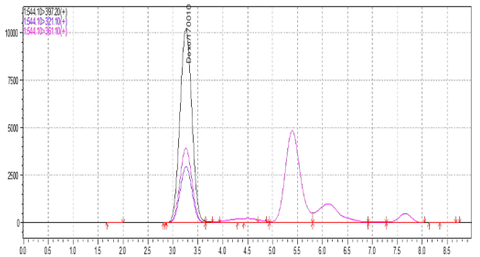
Extract unknown samples and calibration curve

- Protein precipitation (methanol or acetonitrile or mixture)
- Evaporation or dilution
- Reconstitute in MS compatible solution
- Inject into LC/MS

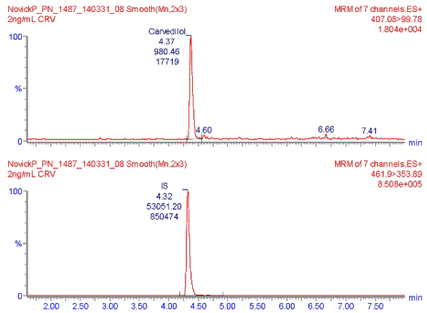
Data Acquisition and Data Analysis

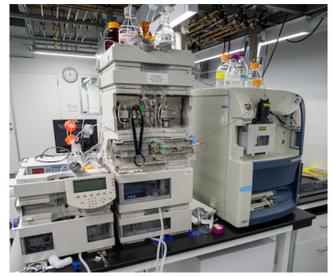
Data Acquisition

LC-MS Data acquired via vendor-specific software



Shimadzu LC/MS8030





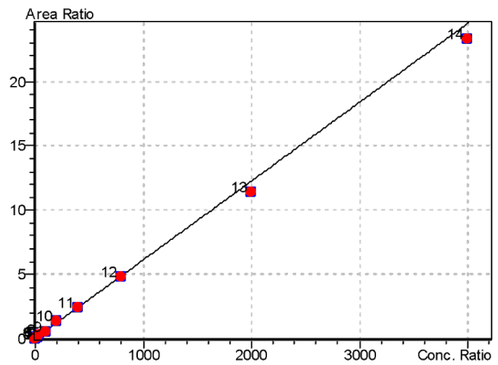
Waters Quattro Premier LC/MS

Data Analysis

ID#	2															
Name	BPV															
	Da	Sample N	Samp	Area	ISTD Area	Area Ratio	Conc.	Dilution F	Accuracy	%Dev	Cal. Point	Sample Ty	Std. Conc.	Level#	S/N	Mark	Ret. Time
1	BP	STD BPV+	0-959	7,068,625	17,415,951	0.405871	65.151	1	-----	-----	-----	Unspiked	0	0	786.86	M	4.161
2	BP	BLK Char	BLK C	63,725	261	244.326	39,841.97	1	-----	-----	-----	Unspiked	0	0	18.69	M	5.684
3	BP	BLK lot2	BLK lo	15,114	73	208.245	33,958.12	1	-----	-----	-----	Unspiked	0	0	15.18	M	6.122
4	BP	BLK/BLK	BLK/B	13,629	92	148.381	24,195.88	1	-----	-----	-----	Unspiked	0	0	14.17	M	6.134
5	BP	BLK/IS	BLK/I	13,358	2,876,067	0.004644	-0.278	1	-----	-----	-----	Unspiked	0	0	9.96	M	6.112
6	BP	STD1	STD1	20,442	2,886,510	0.007082	0.119	1	59.7	-40.3	-----	Standard(	0.2	1	11.48	S	6.112
7	BP	STD2	STD2	29,449	2,984,388	0.009868	0.574	1	143.4	43.42	-----	Standard(	0.4	2	18.76	S	6.12
8	BP	STD3	STD3	32,729	2,912,615	0.011237	0.797	1	99.6	-0.38	*	Standard(	0.8	3	24.22	S	6.124
9	BP	STD4	STD4	55,071	2,982,495	0.018465	1.976	1	98.8	-1.22	*	Standard(	2	4	38.83	V	6.119
10	BP	STD5	STD5	91,407	3,014,138	0.030326	3.91	1	97.7	-2.25	*	Standard(	4	5	72.06	SV	6.117
11	BP	STD6	STD6	182,999	2,971,552	0.061584	9.007	1	112.6	12.59	*	Standard(	8	6	180.22	S	6.12
12	BP	STD14	STD14	70,192,141	3,008,636	23.3302	3,803.50	1	95.1	-4.91	*	Standard(	4000	14	21,073.03	V	6.128
13	BP	STD12	STD12	14,347,979	2,993,804	4.79256	780.503	1	97.6	-2.44	*	Standard(	800	12	3,755.58	V	6.119
14	BP	STD10	STD10	3,886,671	2,923,553	1.32943	215.76	1	107.9	7.88	*	Standard(	200	10	1,428.48	V	6.119
15	BP	STD8	STD8	866,754	2,996,486	0.289257	46.135	1	115.3	15.34	*	Standard(	40	8	411.78	M	6.114
16	BP	BLK/BLK	BLK/B	13,935	2,239	6.22365	1,013.88	1	-----	-----	-----	Unspiked	0	0	6.57	M	6.131
17	BP	BLK	BLK	13,881	980	14.1581	2,307.77	1	-----	-----	-----	Unspiked	0	0	8.09	M	6.163
18	BP	Sample 1	DMP(	6,024,925	3,175,227	1.89748	770.981	2.5	-----	-----	-----	Unknown	-----	0	3,363.13		6.127
19	BP	Sample 1	DMP(	6,781,875	2,941,674	2.30545	374.921	1	-----	-----	-----	Unknown	-----	0	3,042.79	SV	6.12

$Y = (0.00612480)X + (0.00637299)$

$R^2 > 0.99$



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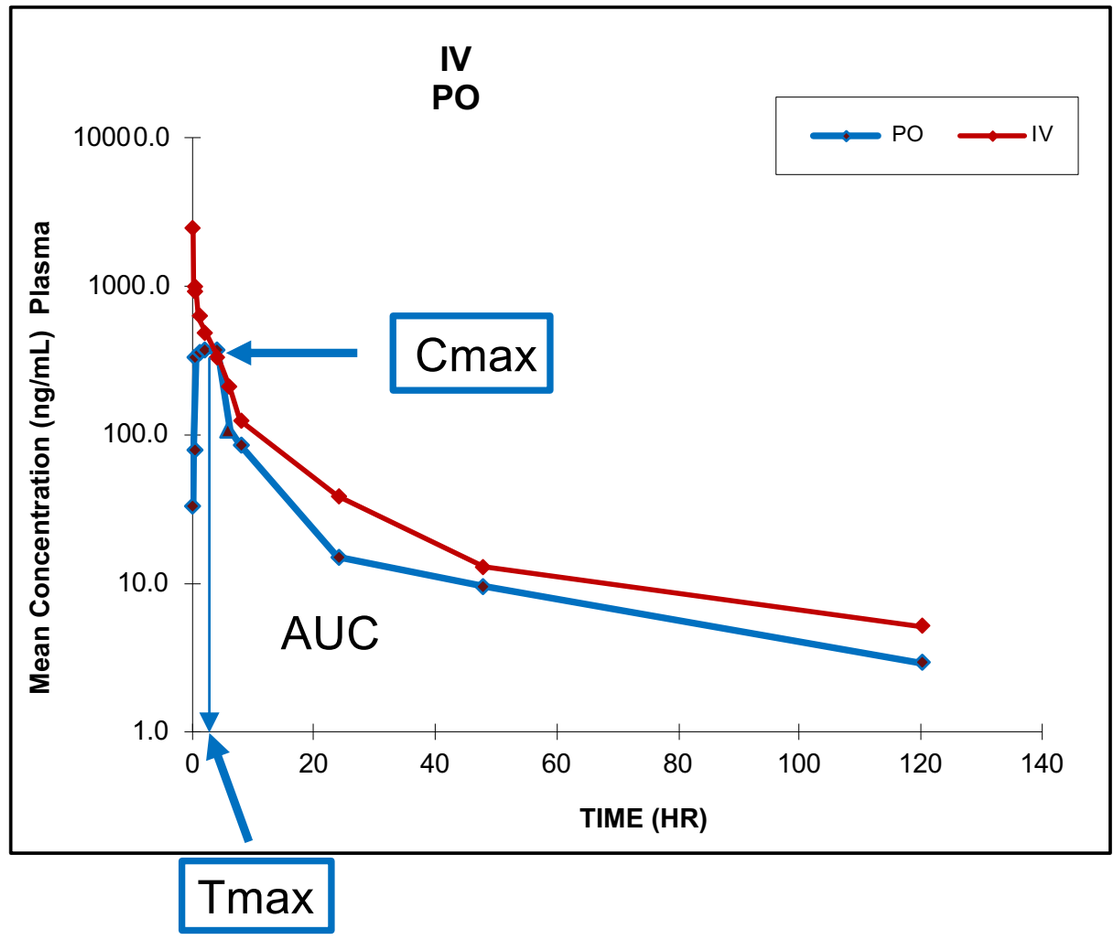
Plasma Concentration-time Profiles and Bioavailability

PK parameters

	IV	PO
Tmax (hr)	0.0833	2
Cmax (ng/ml)	2467	370
T1/2 (hr)	12	13
AUC(total)	5885	3701
Cl (L/kg/hr)	1	
VdB (L/kg)	15	
Bioavailability(%)		**63**

Bioavailability(%)=AUC(total) PO/AUC(total) IV

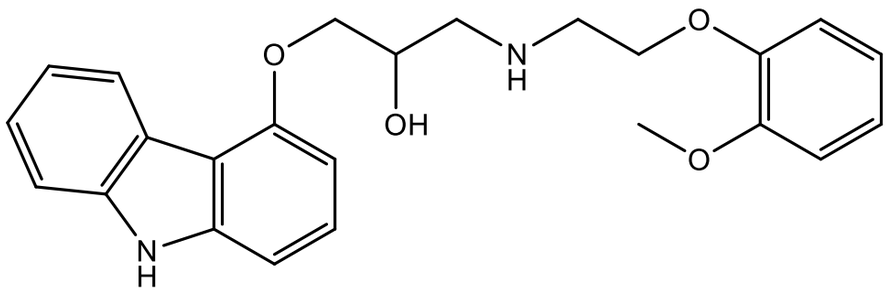


PK project Example

Quantitation of Carvedilol (CRV) in mouse plasma

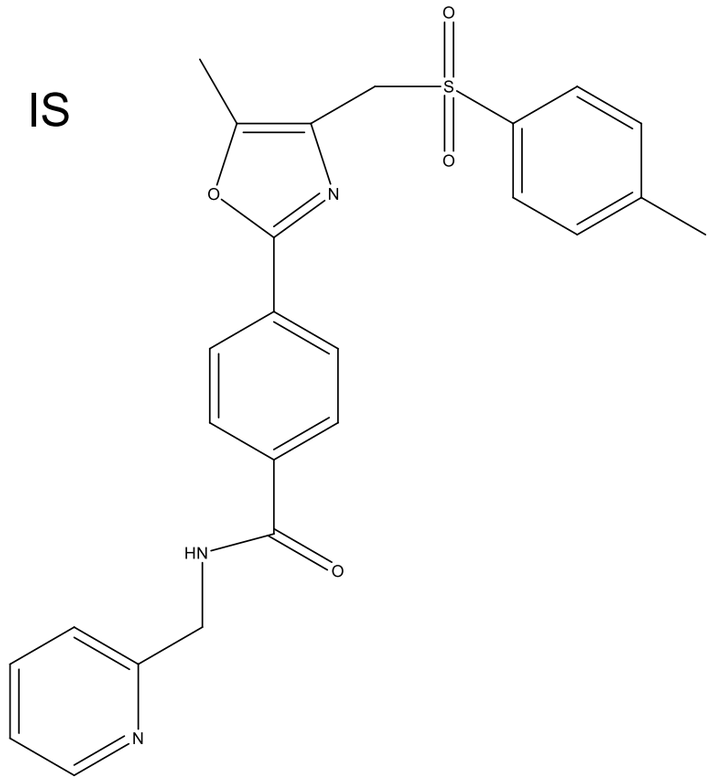
Structures of Analyte and Internal Standard

Carvedilol



Chemical Formula: $C_{24}H_{26}N_2O_4$
Exact Mass: 406.1893
Molecular Weight: 406.4742

IS



Chemical Formula: $C_{25}H_{23}N_3O_4S$
Exact Mass: 461.1409
Molecular Weight: 461.5328

LC/MS/MS Method

Compound Name	SRM Transition	Dwell (s)	Cone (V)	Collision (V)
Carvedilol	407>100	0.02	30	25
IS	462>354	0.02	30	25

- Agilent HPLC 1100 pump
- Micromass Quattro triple quadrupole mass spectrometer
- ESI+
- Column: Polaris A 5um C18 50x2.1mm
- Run time 8 min
- Injection volume – 10uL
- CRV RT=4.36min
- IS RT=4.31min

Time (min)	A% (0.1% Formic acid in water	B% (0.1% Formic Acid in acetonitrile)	Flow rate (ml/min)
0	85	15	0.25
0.5	85	15	0.25
3.5	5	95	0.25
5	5	95	0.25
5.1	85	15	0.25
8	85	15	0.25

Carvedilol: Standard Solutions

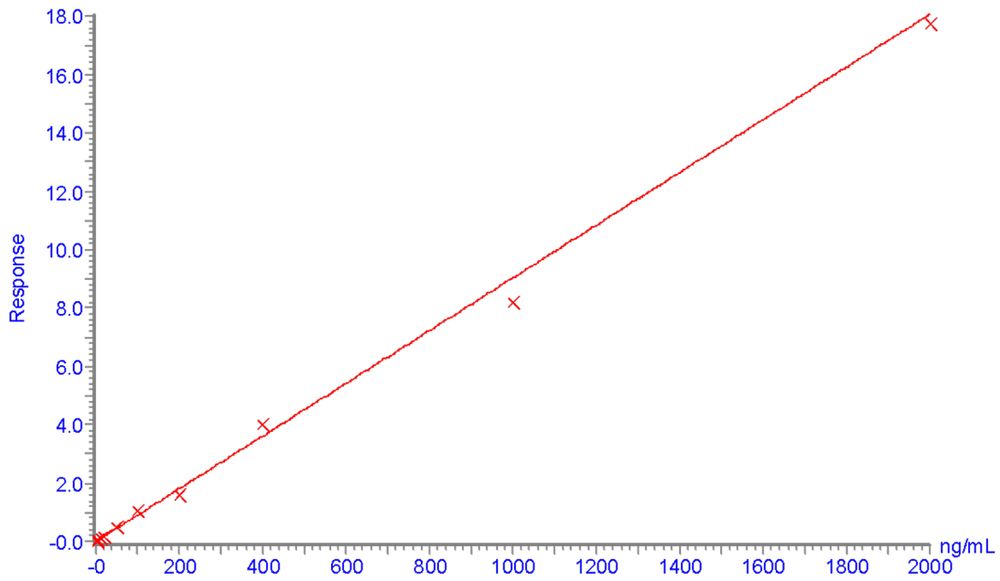
Spiking solution concentration (ng/mL)	plasma volume (uL)	Amount spiked (uL)	Final plasma concentration (ng/mL)
10	50	10	2
10	50	20	4
50	50	10	10
50	50	20	20
250	50	10	50
250	50	20	100
1000	50	10	200
1000	50	20	400
5000	50	10	1000
5000	50	20	2000

- 50uL plasma
- 10 or 20uL Standard Spiking solution
- 20uL IS (400ng/mL)
- 200uL ice cold methanol
- Vortex, incubate for 1 hr at -20°C
- Vortex, centrifuge transfer to injection vial

Carvedilol Calibration Curve

Calibration Curve

Compound name: Carvedilol
Correlation coefficient: r = 0.993313, r^2 = 0.986671
Calibration curve: 0.0090363 * x + 6.7961e-005
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x^2, Axis trans: None



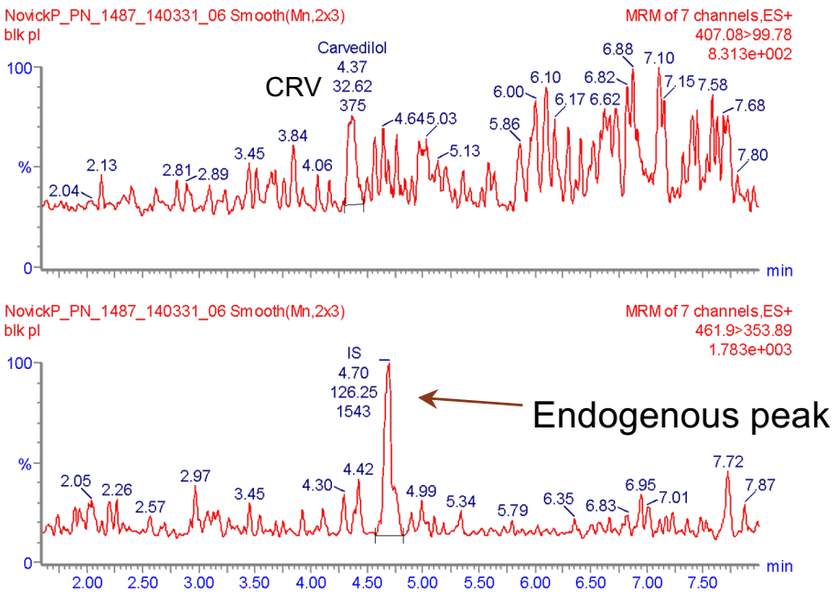
2ng/mL – 2000ng/mL

Standards back calculated concentrations

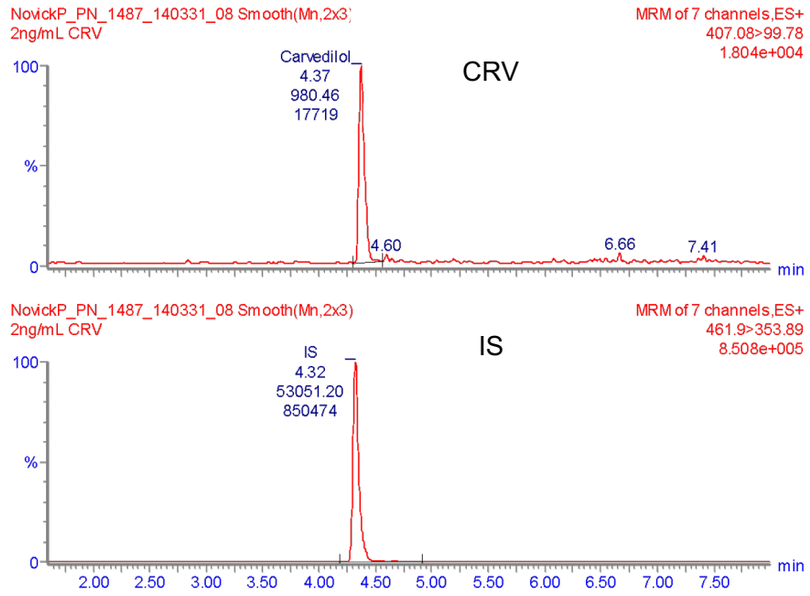
Sample name	Actual concentrations (ng/mL)	Measured concentrations (ng/mL)	% difference (measured/actual)
2ng/mL CRV	2	2.04	1.9
4ng/mL CRV	4	3.90	-2.6
10ng/mL CRV	10	10.1	1.3
20ng/mL CRV	20	16.9	-15.5
50ng/mL CRV	50	56.2	12.4
100ng/mL CRV	100	114	14.3
200ng/mL CRV	200	176	-12.2
400ng/mL CRV	400	446	11.6
1000ng/mL CRV	1000	905	-9.5
2000ng/mL CRV	2000	1965	-1.8

LC/MS Chromatograms

Blank Plasma



Standard 2 ng/mL



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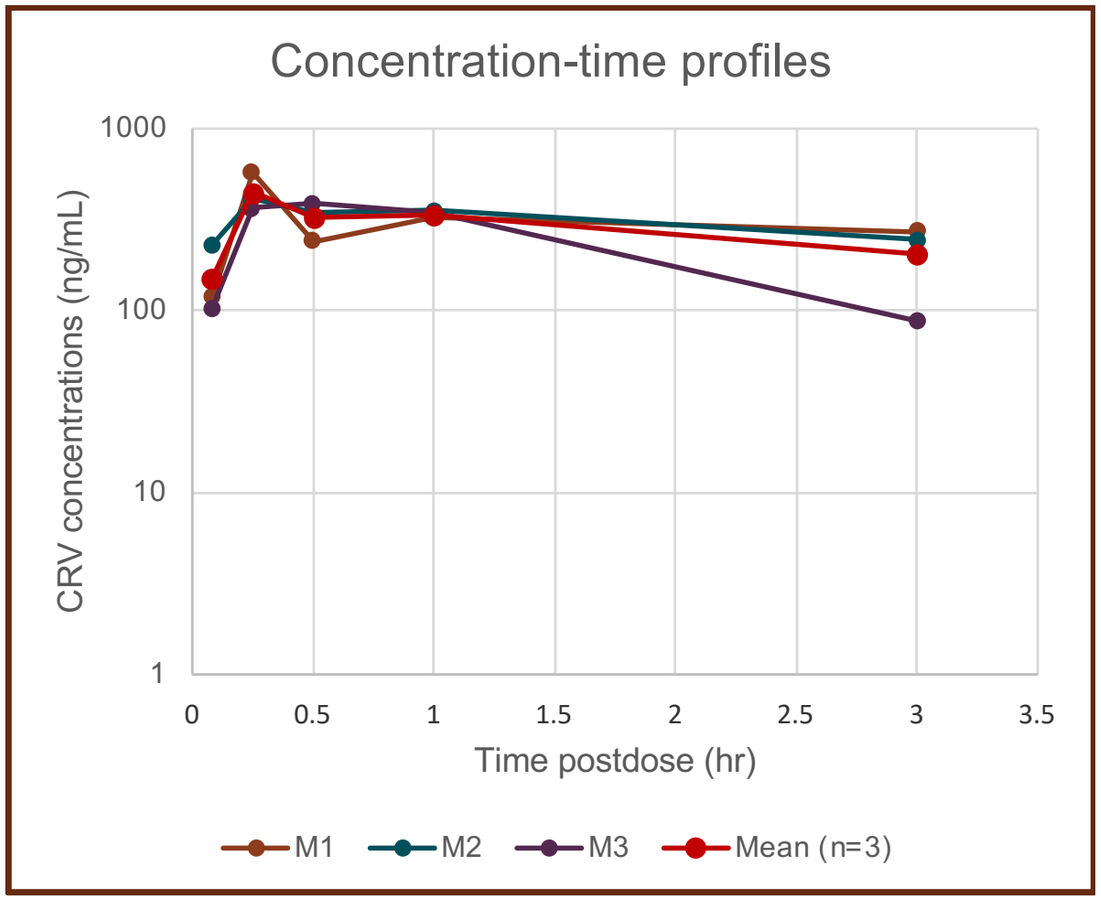
Results: CRV Concentration-Time Profile in Plasma



		ng/mL		
Time post dose (hr)	M1	M2	M3	Mean (n=3)
0.083	119	230	103	151
0.25	573	412	363	450
0.5	241	343	389	324
1	328	351	342	340
3	274	246	88	203
AUC (ng*hr/mL)	909	929	749	862

Conclusion

- It is common practice to use LC/MS assays to support high throughput screening of large compound libraries against biological targets hypothesized to be effective in humans
- LC/MS is an essential tool to support metabolism, PK/PD and toxicology studies throughout drug discovery and development
- Due to high sensitivity, selectivity and specificity, LC/MS methodology is used extensively in clinical phases
- The multi-stage drug discovery and development process is challenging and rewarding at the same time

References

Literature:

- Mass Spectrometry: Principles and Applications by E. de Hoffmann, V. Stroobant
- Mass spectrometry in drug metabolism and Pharmacokinetics by R. Ramanathan
- LC/MS: A practical User's Guide by M. McMaster
- Handbook of drug metabolism by T. F. Woolf

Standards:

- Cambridge Isotope Labs - www.isotope.com
- Cerriliant - www.cerilliant.com
- Anaspec – www.anaspec.com
- Sigma-Aldrich – www.sigmaaldrich.com

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SUMS Group

People

Applications

Director – Allis Chien, Ph.D.

Proteomics

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Fang Liu, PhD
Rowan Matney, BA
Kratika Singhal, MS

Protein ID
Quantitative – PRM, TMT
Protein interactions
Protein structure
Phosphoproteomics, PTMs

Small Molecule

Ludmila Alexandrova, PhD
Karolina Krasinska, MS
Beryl Xia, PhD
(Theresa McLaughlin, MS)

Absolute quantitation
Targeted metabolomics
PK, Stability studies
Metabolite ID
Intact protein

Open Access

Theresa McLaughlin, MS
(Beryl Xia, PhD)

GC/MS
LC/MS
HRMS
MALDI-TOF

Accounting Associate – William Lee, BS

Details & registration: mass-spec.stanford.edu/events

Stay tuned

More webinars on Thursdays, noon-1pm Pacific

Requests welcome!

May 28, 2020

Mass spec bloopers! And other amusing adventures

Birgit Schilling, Buck Institute
David Quilici, University of Nevada, Reno
Brett Phinney, University of California, Davis
Kimberly Lee, Cell Signaling Technology
Toni Koller, Massachusetts Institute of Technology
Allis Chien, Stanford University

June 18, 2020

ASMS review: recap the reboot!

June 25, 2020

Fundamentals: **Bioanalytical LC/MS method validation – fit for purpose**

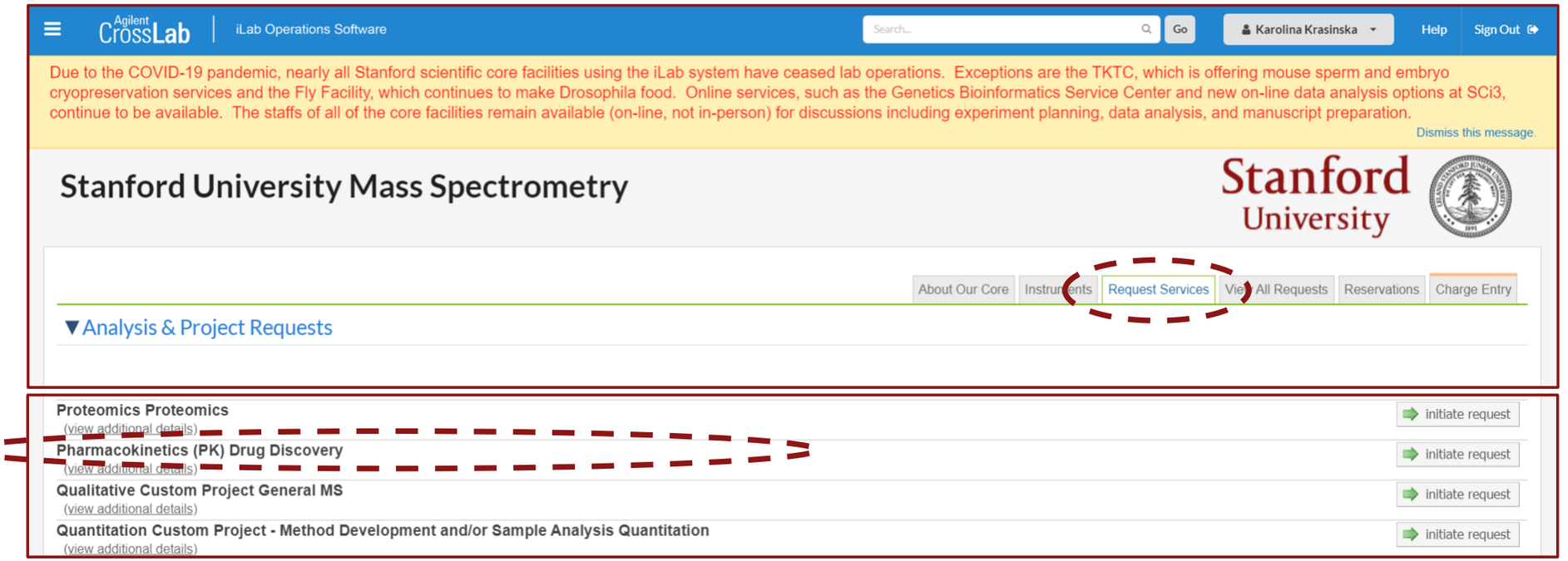
Ludmila Alexandrova, PhD

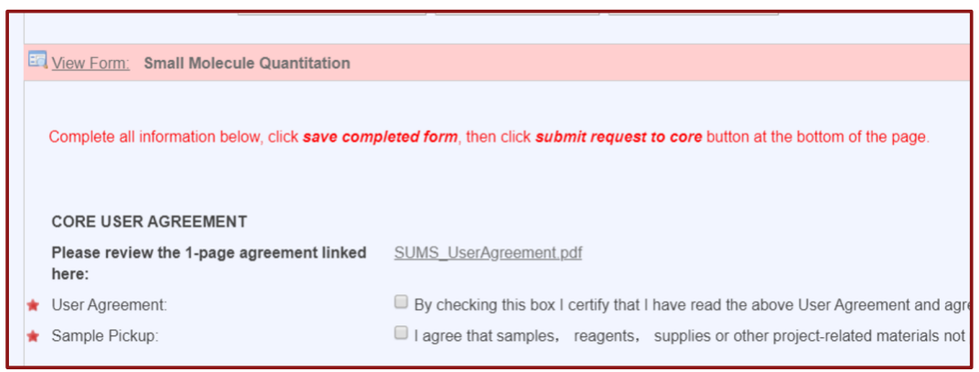
June 1-12 – Join the ASMS Reboot!

https://asms.org/conferences/asms-2020-reboot/

http://mass-spec.stanford.edu

We are Stanford University Core Lab





iLab help:

- SUMS website
- William Lee