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Challenges of LC-MS/MS method development for the quantitation of a polar low molecular weight biomarker in biological fluids

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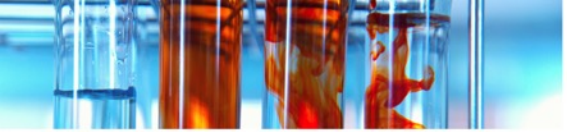
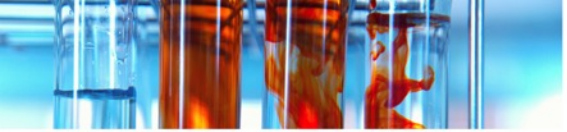


Agenda

- Introduction to small molecule biomarkers
- Challenges with LC-MS/MS assay development for urea
- Experimental optimization & final conditions
- Validation Data
- Conclusions



LC-MS/MS Challenges of Low Molecular Weight Analytes

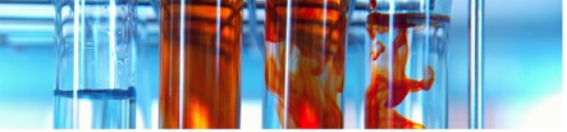
Factors affecting quantitation of low molecular weight analytes (<100 Da) in biological matrices

- Few chemical groups within molecule
  - Limited number of options for product ions
  - Product ion transitions are in turn likely to be simple and non-specific.

- High background & chromatographic interferences from ions from matrix and environmental contaminants
- Can lead to high baseline in low molecular weight analyte assays (Potential LLOQ impact)
  - Methanol – M_r = 32.04 Da
  - Acetonitrile – M_r = 41.05 Da
  - Formic Acid – M_r = 46.03 Da
  - Acetic Acid – M_r = 60.05 Da

Monoisotopic ion mass (singly charged)	Ion type	Formula for M or subunit or sequence	Compound ID or species	Possible origin and other comments
33,033491	$[M+H]^+$	CH_3OH	Methanol	Acetonitrile, solvent
42,033825	$[M+H]^+$	CH_3CN	Acetonitrile	Acetonitrile, solvent
59,060374	$[M+NH_4]^+$	CH_3CN	Acetonitrile	Acetonitrile, solvent
63,044056	$[A_1B+H]^+$	$[C_2H_4O]_nH_2O$	PEG	Polyethylene glycol, ubiquitous polyether
64,015769	$[M+Na]^+$	CH_3CN	Acetonitrile	Acetonitrile, solvent
65,059706	$[M_2+H]^+$	CH_3OH	Methanol	Methanol, solvent

**Keller, B.O.; Sui, J.; Young, A.B.; Whittal, R.M. Interferences and contaminants encountered in modern mass spectrometry (Analytica Chimica Acta (Review/tutorial, Special Issue on Mass Spectrometry), 2008)*

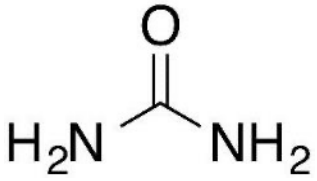


Biomarker Analysis using LC-MS/MS

- Fundamental issue is that biomarkers typically have an endogenous presence
- Simple spiking of standards into biological matrices to make calibration curves may not always be feasible.
- Assessment of different approaches for small molecule biomarkers
  - Standard addition to baseline endogenous concentration
  - Use of stable labeled heavy (surrogate) standards for the analyte of interest.
  - Use of an alternative interference-free matrix (surrogate) for calibration standards preparation.
    - Core assay standards and QCs are typically made in the surrogate along with QC samples prepared in the actual matrix to mimic study samples.
    - Development includes the assessment of parallelism between candidate surrogates and true matrix to determine best choice.

Urea as a Target Biomarker

- Low molecular weight (M_r = 60.06 Da)
  - Rapid and uniform diffusion across the peripheral blood and epithelial lining.
  - Urea diffuses freely throughout the body and is minimally affected by disease states



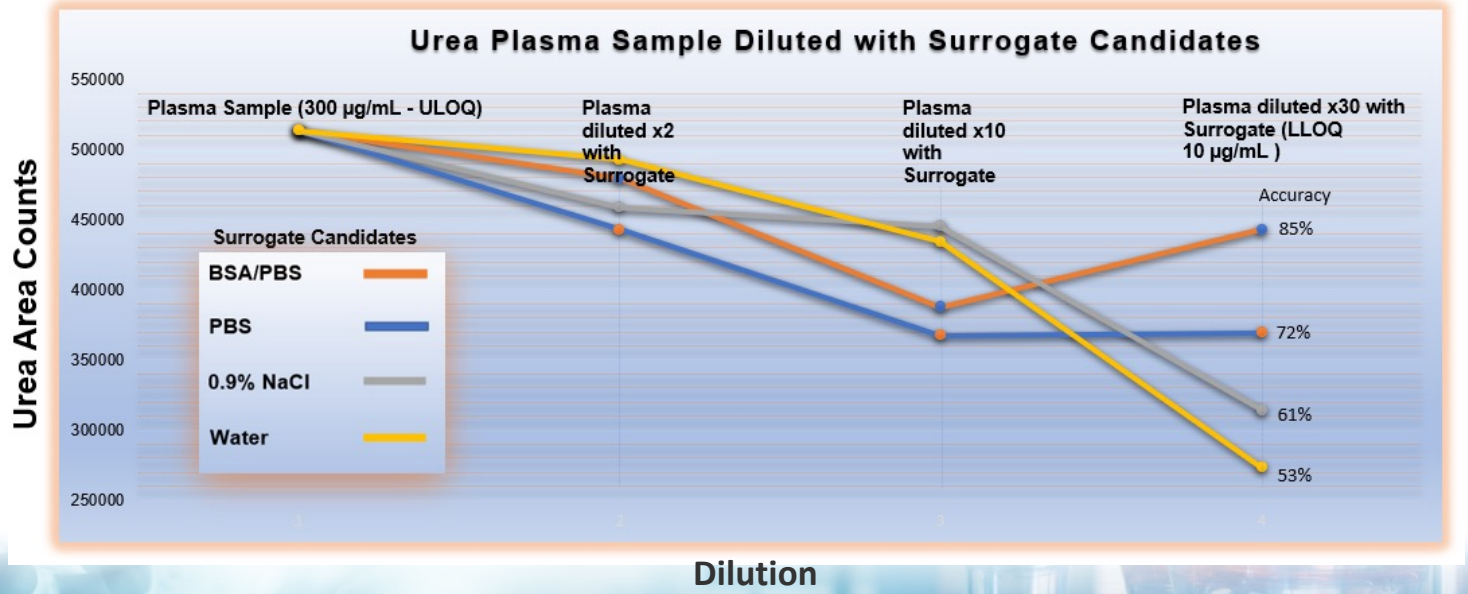
- Endogenous urea measurement in plasma often utilized as a control marker
  - Accounts for dilutions during sample collection or processing > dilution marker for volume normalization of biological matrices
  - Applicable when study includes samples from different matrices within the same project.

- In this case study, urea was surrogate for administered antibiotic (drug) with intracellular action. Measurements in plasma, BAL (BronchoAlveolar Lavage) & lung lining cells were used to normalize assessment of drug penetration

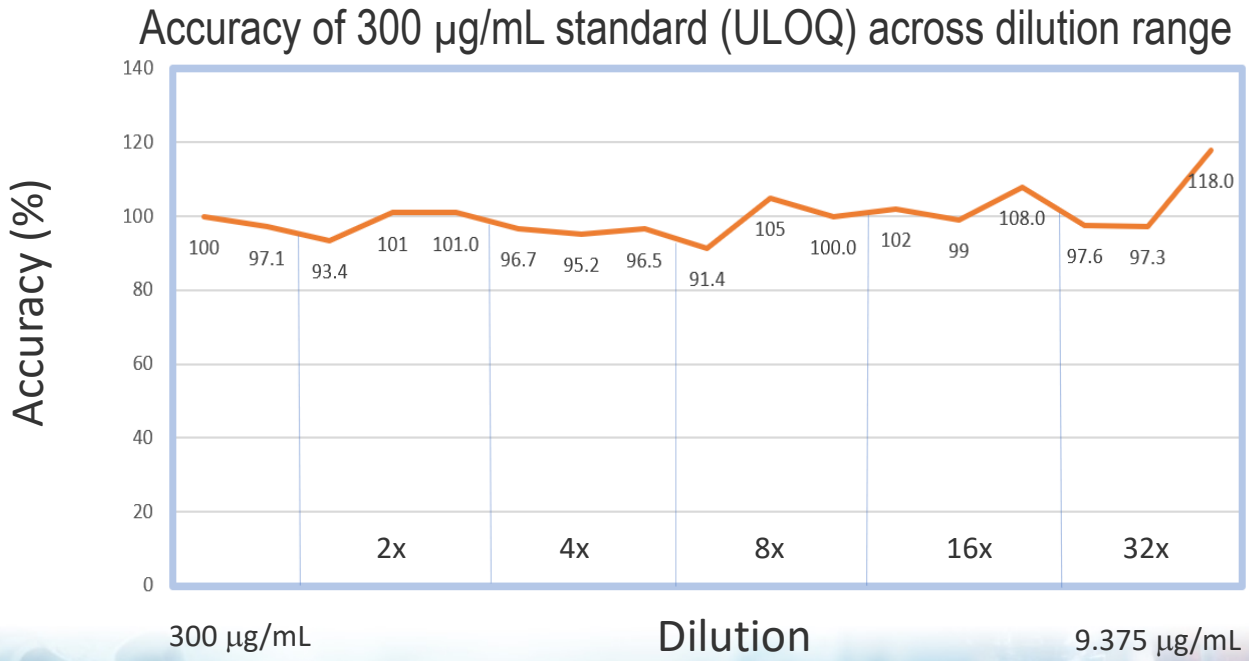
- **High endogenous urea concentrations (~ 275 µg/mL) in plasma, combined with its low molecular weight and highly polar properties, are significant barriers to simple LC-MS/MS approaches**

Monitoring Potential Surrogate Matrices (Plasma)

Effect of dilution of the true matrix with surrogate based on accuracy of measurement for urea from the upper limit (ULOQ) to the lower limit (LLOQ) of the assay

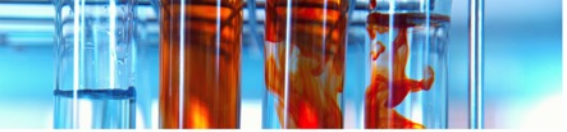


Effect of Dilution in the Selected Surrogate Matrix



Surrogate Matrix

10 g bovine serum albumin (BSA) in 140 mL phosphate buffered saline

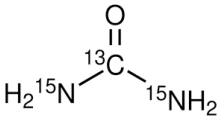


Extraction Challenges

- Conventional protein precipitation with organic solvents initially assessed
- Hydrophilic nature prevents quantitative distribution into acetonitrile from more aqueous environment
- Low Sensitivity – inability to hit requested LLOQ
- Methanol extraction showed low background but poor chromatographic performance (Loss of signal)

Optimized Urea Extraction

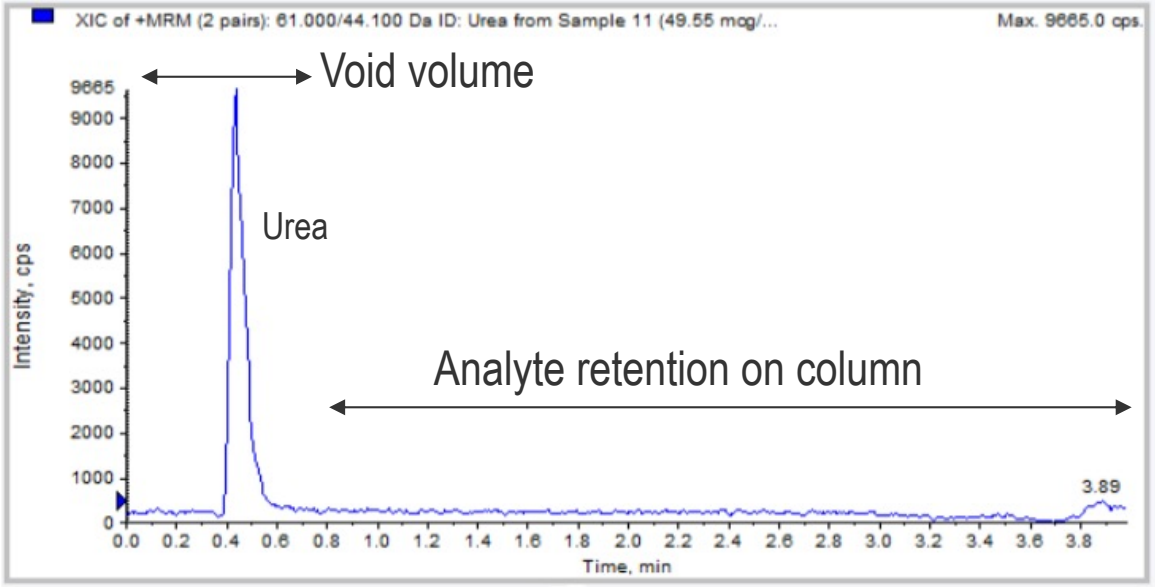
- Aliquot 100 µL calibration standard, QC or sample
  - Plasma or BSA/PBS (10 g/140 mL)
- Addition of stable-labeled internal standard (25 µL, 1 mg/mL in H_2O)
- Precipitation with 30% trichloroacetic acid TCA in water
- Vortex (15 minutes) & centrifuge samples (15 minutes)
- Transfer 25 µL aliquot to fresh tubes
- Dilution with acetonitrile (650 µL)
- Vortex (10 minutes)
- Transfer to chilled autosampler
- Injection (20 µL) onto UHPLC system



Integra Viaflo 96

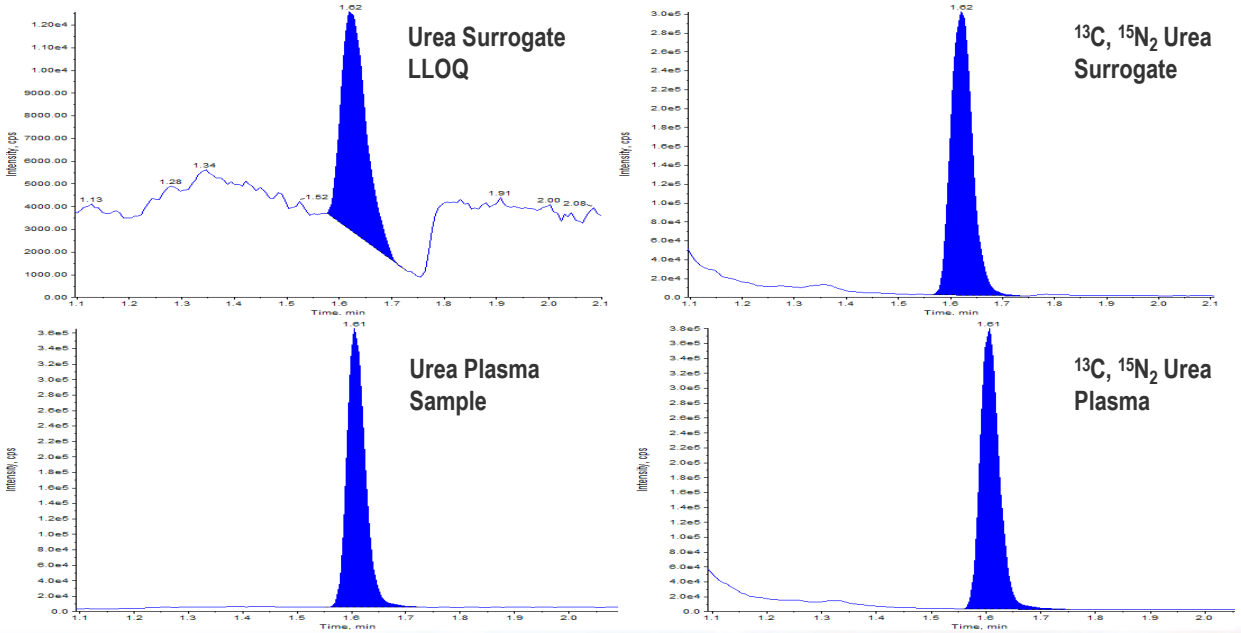
Chromatographic Optimization – Reversed Phase

- Reversed phase chromatography proved unsuccessful
- Limited retention
- Background Interference & Ion suppression



Chromatographic Optimization - HILIC

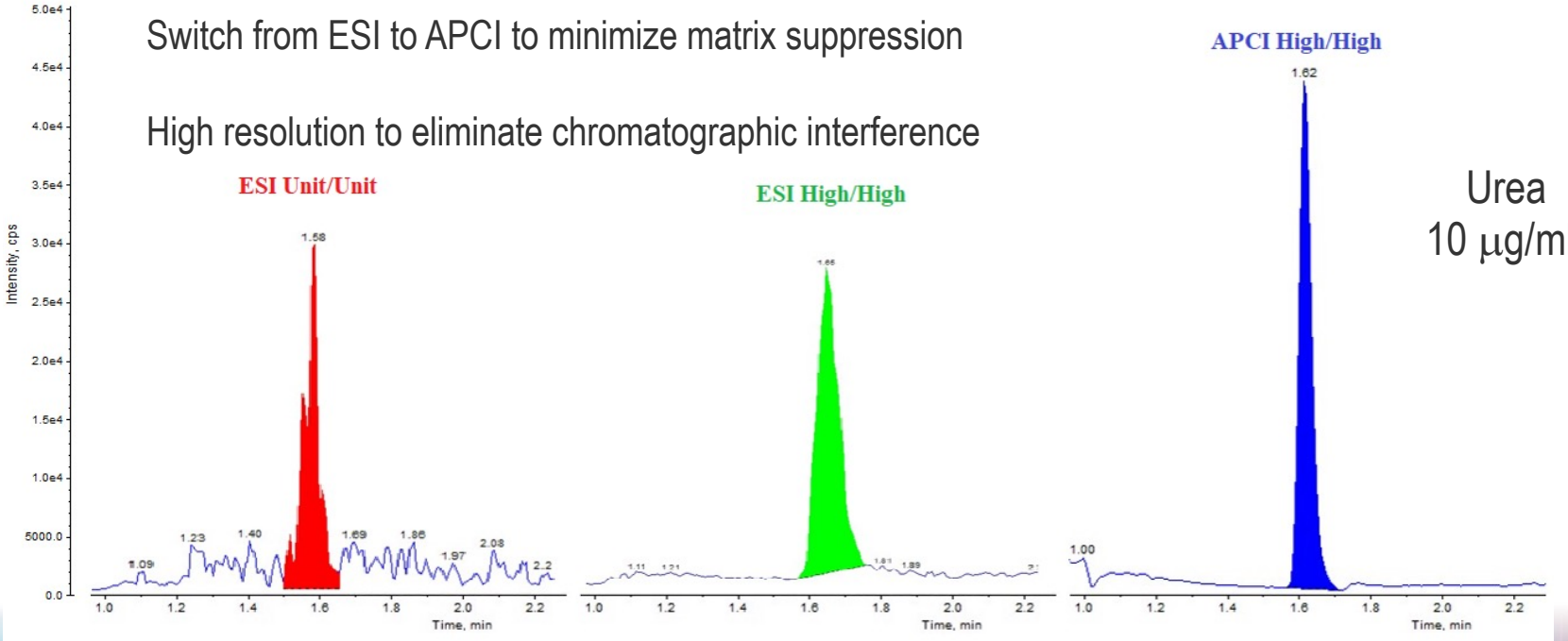
- Hydrophilic Interaction Chromatography (HILIC) Approach
- Analyte and IS well resolved from matrix components (k' ~ 5)
- Residual trichloroacetic acid from extraction aided analyte retention



MS Optimization: ESI vs APCI

Switch from ESI to APCI to minimize matrix suppression

High resolution to eliminate chromatographic interference



Urea
10 µg/mL

LC-MS/MS Conditions

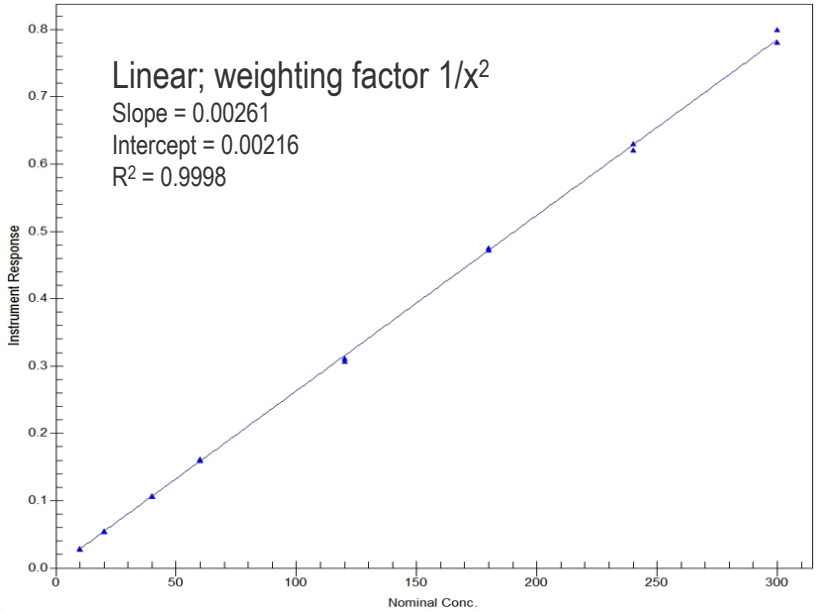
- Shimadzu LC-30 System
- Shimadzu SIL-30AC Autosampler at 4 °C
- Waters XBridge BEH Amide (100 x 2.1 mm) at 45 °C
- Gradient LC Conditions
- Mobile Phase A:- 10 mM Ammonium Acetate in Deionized water
- Mobile Phase B:- Acetonitrile
- Flow Rate 0.800 mL/min

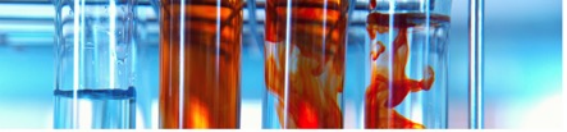
- Retention Time
- Urea 1.62 min
- ^{13}C, $^{15}N_2$ Urea 1.62 min

- SCIEX 6500+ QTRAP
- APCI in positive ion mode
- Q1 High Resolution, Q3 High Resolution
- Selected Reaction Monitoring (SRM)
- Urea m/z = 61.054 to m/z = 44.100
- ^{13}C, $^{15}N_2$ Urea m/z = 64.035 to m/z = 46.000

Typical Calibration Curve (Surrogate Matrix)

Cal.Std. (µg/mL)	Measured Concentration	%DEV
10.0	10.0	0.0
	9.97	-0.3
20.0	20.0	0.0
	19.9	-0.5
40.0	40.0	0.0
	40.4	1.0
60.0	60.3	0.5
	61.1	1.8
120	117	-2.5
	118	-1.7
180	180	0.0
	181	0.6
240	237	-1.3
	241	0.4
300	298	-0.7
	305	1.7





Precision & Accuracy of QCs

Surrogate Matrix

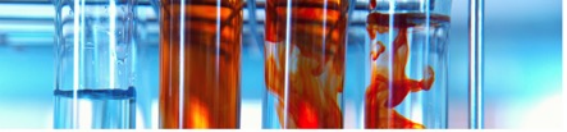
QC Level	LLOQ QC	QC_L	QC_M	QC_H
Conc. (µg/mL)	10.0	30.0	90.0	225
A&P 1	10.3	31.9	91.6	235
	10.8	32	92.5	233
	10.2	31.1	94.4	235
	11.6	32	90.2	234
	10.1	31.9	91.3	231
	10.5	31.6	91.9	239
Mean	10.6	31.8	91.9	235
%RSD	5.2	1.1	1.5	1.1
%DEV	6.0	6.0	2.1	4.4
A&P 2	11.8	32.6	94.5	244
	11.1	33.0	96.1	243
	11.0	32.5	97.2	240
	11.1	32.6	97.3	241
	11.2	33.0	96.3	249
	11.5	32.1	97.9	246
Mean	11.3	32.6	96.6	244
%RSD	2.7	1.0	1.3	1.4
%DEV	13.0	8.7	7.3	8.4
A&P 3	9.77	29.5	89.4	231
	9.75	30.0	90.3	232
	10.2	29.5	90.8	229
	10.3	29.9	89.4	230
	9.97	30.5	90.4	231
	10.1	29.8	90.1	230
Mean	10.0	29.9	90.1	231
%RSD	2.3	1.2	0.6	0.5
%DEV	0.0	-0.3	0.1	2.7
Mean (Inter)	10.6	31.4	92.9	236
%RSD (Inter)	6.1	3.9	3.2	2.6
%DEV (Inter)	6.0	4.7	3.2	4.9

Plasma

QC Level	QC_L	QC_L	QC_M	QC_M	QC_H	QC_H
Conc. (µg/mL)	36	60.6	152	180	291	303
A&P 1	35.2			182	292	
	36.8			180	291	
	35.0			177	292	
	35.7			180	302	
	36.7			183	290	
	35.4			179	297	
Mean	35.8			180	294	
%RSD	2.2			1.2	1.6	
%DEV	-0.6			0.0	1.0	
A&P 2		58.8	153			303
		59.5	152			304
		60.2	153			304
		61.2	151			303
		60.4	151			299
		60.3	153			305
Mean		60.1	152			303
%RSD		1.4	0.6			0.7
%DEV		-0.8	0.0			0.0
A&P 3		56.5	142			281
		56.9	142			286
		57.6	140			284
		57.8	143			288
		57.4	143			280
		56.7	143			289
Mean		57.2	142			285
%RSD		0.9	0.8			1.3
%DEV		-5.6	-6.6			-5.9
Mean (Inter)		58.6	147			294
%RSD (Inter)		2.8	3.6			3.4
%DEV (Inter)		-3.3	-3.3			-3.0
n		12	12			12

Plasma dilution with surrogate matrix

QC Level (µg/mL)	300	1010	2670
Dilution	2x	5x	10x
	303	1040	2680
	302	1020	2550
	300	1030	2600
	302	1060	2760
	304	1050	2590
	301	1050	2670
Mean	302	1040	2640
%RSD	0.5	1.4	2.9
%DEV	-0.3	3	-1.1



Method Development in BAL Supernatant & Cells

- Sourcing of true matrix difficult so challenges primarily logistical
  - Limited suppliers
  - Cell harvesting challenging

- Bovine serum albumin (BSA) in 140 mL phosphate buffered saline surrogate matrix not applicable to BAL & cell matrix

- Saline (0.9% NaCl in water) used as a surrogate because BAL fluid is mostly a collection of saline.

- Downstream analytical conditions consistent with plasma approach

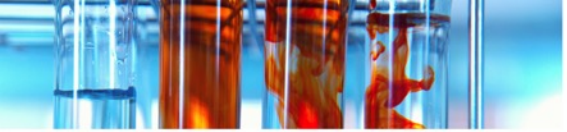
Analytical Performance in Human Bronchoalveolar Lavage (BAL)

Surrogate Matrix (0.9% Saline)

	Curve Number	QCS 0.75 0.750 µg/mL	%Bias	QCS 2.25 2.25 µg/mL	%Bias	QCS 9 9.00 µg/mL	%Bias	QCS 22.5 22.5 µg/mL	%Bias
	A&P 1	#0.923	23.1	1.98	-12.0	8.62	-4.2	23.4	4.0
		0.898	19.7	1.96	-12.9	9.26	2.9	23.0	2.2
		0.873	16.4	##1.85	-17.8	9.22	2.4	23.9	6.2
		0.827	10.3	2.07	-8.0	9.03	0.3	23.0	2.2
		0.896	19.5	2.01	-10.7	9.04	0.4	23.2	3.1
		0.840	12.0	##1.83	-18.7	8.83	-1.9	23.4	4.0
	A&P 2	0.792	5.6	2.10	-6.7	9.12	1.3	22.9	1.8
		0.747	-0.4	2.26	0.4	8.72	-3.1	23.5	4.4
		0.735	-2.0	2.14	-4.9	9.12	1.3	22.8	1.3
		0.742	-1.1	2.27	0.9	8.95	-0.6	22.9	1.8
		0.806	7.5	2.23	-0.9	9.16	1.8	23.4	4.0
		#1.01	34.7	2.11	-6.2	9.14	1.6	22.9	1.8
	A&P 3	0.709	-5.5	2.28	1.3	8.76	-2.7	22.7	0.9
		0.774	3.2	2.22	-1.3	9.34	3.8	22.9	1.8
		0.765	2.0	2.23	-0.9	9.15	1.7	22.2	-1.3
		0.781	4.1	2.14	-4.9	8.93	-0.8	23.6	4.9
		0.745	-0.7	2.17	-3.6	9.17	1.9	23.0	2.2
		0.752	0.3	2.26	0.4	9.03	0.3	23.2	3.1
Mean Concentration (µg/mL)		0.812		2.12		9.03		23.1	
Inter-run SD		0.0803		0.142		0.197		0.390	
Inter-run %CV		9.9		6.7		2.2		1.7	
Inter-run %Bias		8.3		-5.8		0.3		2.7	
n		18		18		18		18	
# > 20%Bias									
## > 15%Bias									

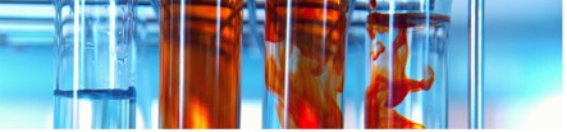
BAL Fluid

	Curve Number	True QCS low 160 µg/mL	%Bias	True QCS mid 170 µg/mL	%Bias	True QCS high 173 µg/mL	%Bias
	A&P 1	167	4.4	179	5.3	167	-3.5
		159	-0.6	178	4.7	180	4.0
		150	-6.3	175	2.9	184	6.4
		169	5.6	171	0.6	169	-2.3
		156	-2.5	156	-8.2	181	4.6
		157	-1.9	161	-5.3	159	-8.1
	A&P 2	143	-10.6	152	-10.6	160	-7.5
		147	-8.1	157	-7.6	158	-8.7
		162	1.3	155	-8.8	163	-5.8
		147	-8.1	163	-4.1	##145	-16.2
		154	-3.8	163	-4.1	162	-6.4
		147	-8.1	165	-2.9	154	-11.0
	A&P 3	157	-1.9	163	-4.1	157	-9.2
		152	-5.0	164	-3.5	160	-7.5
		161	0.6	168	-1.2	159	-8.1
		156	-2.5	167	-1.8	168	-2.9
		152	-5.0	161	-5.3	152	-12.1
		149	-6.9	158	-7.1	162	-6.4
Mean Concentration (µg/mL)		155		164		163	
Inter-run SD		7.14		7.71		10.2	
Inter-run %CV		4.6		4.7		6.3	
Inter-run %Bias		-3.1		-3.5		-5.8	
n		18		18		18	
# > 20%Bias							
## > 15%Bias							



Conclusions

- Small molecule biomarkers can be challenging analytes due to potential for interference
- Polar nature of urea meant usually adopted approaches for extraction, chromatography and mass spectrometry needed careful optimization
- High throughput assay for the direct measurement of urea was successfully validated and used to support analysis of plasma & bronchoalveolar lavage samples from a clinical study
- Routine analysis is possible but regular instrument cleaning is essential due to build up of matrix interference.



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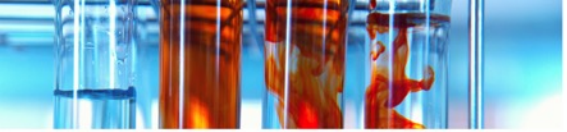
- Disclaimer: This talk reflects the views of the authors and should not be construed to represent NIAID's views or policies.



Thanks for your attention

Questions ?