Overcoming Triple Quadrupole Selectivity Challenges by Use of High-Resolution Accurate Mass Spectrometry to Enable Sensitive Quantitation of a Small Molecule Therapeutic in Rabbit Plasma

Daniel Mulvana[1], Robert Sturm[1], Chet Bowen[2], John Buckholz[1], Raymond Biondolillo[1], Kathlyn McCardle[1], Chris Evans[2], Barry Jones[1]

[1]Q[2] Solutions, 19 Brown Road, Ithaca, NY, USA. [2]GlaxoSmithKline, King of Prussia, PA, USA.

Q² Solutions
a Quintiles Quest Joint Venture

Overview

Purpose:
- Validated triple quadrupole rat and human plasma methods were used as starting points for the development of a rabbit plasma method for the small molecule GSKA. The lower limit of quantitation (LLOQ) for the rat plasma method was 250 pg/mL using 25 µL of plasma and the LLOQ for the human plasma method was 10 pg/mL using 200 µL of plasma.
- A lower LLOQ (1 pg/mL) was desired for the rabbit plasma method; however, interfering chemical noise due to isobaric interference hindered this effort.
- High-resolution accurate mass spectrometry (HRAMS) was investigated as an alternative technique to enable higher sensitivity by increased selectivity.

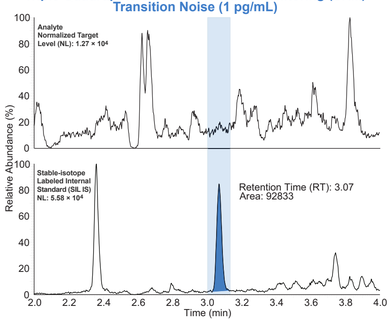
Method:
- GSKA was extracted from rabbit plasma using a double liquid-liquid extraction.
- Chromatographic separation was performed by ultra-high-performance liquid chromatography (UHPLC).
- Heated electrospray ionization (HESI) was used for detection on a Q Exactive (Thermo Scientific) mass spectrometer. Higher energy collision-induced dissociation (HCD) was used to enable tandem mass spectrometric (MS/MS) detection.

Results:
- An LLOQ of 2 pg/mL using 200 µL of rabbit plasma was achieved.
- The method was validated to full regulatory compliance.

Introduction

- Triple quadrupole rat and human plasma methods for the small molecule GSKA were challenged by high chemical noise due to isobaric interference. Efforts to generate cleaner extracts included double liquid-liquid extraction with methyl *tert*-butyl ether (MTBE) and pre-rinsing of the 96-well plate with extraction solvent.

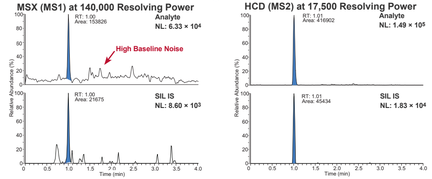
Triple Quadrupole Selected Reaction Monitoring (SRM) Transition Noise (1 pg/mL)



- HRAMS using a Q Exactive was investigated as an alternative technique. Initial efforts using MS1 multiplexed targeted selected ion monitoring (MSX) were unsuccessful due to the isobaric interference, even at 140,000 resolving power.
- Improved selectivity, and thereby sensitivity, was achieved using HCD to enable MS/MS detection.
- Using HCD and 80 µL of rabbit plasma, a 10 pg/mL LLOQ was achieved. Additional method refinement enabled a LLOQ of 2 pg/mL using 200 µL of rabbit plasma.

	Exact Mass	[M+H]⁺
GSKA	390.065 Da	*m/z* 391.073
SIL IS	397.088 Da	*m/z* 398.096

Targeted Multiplexed MSX (MS1) vs HCD (MS2) on the Q Exactive (2.5 ng/mL)



- High baseline noise from an isobaric interferent was observed in the analyte extracted-ion chromatogram (XIC) channel even when a resolving power of 140,000 was used.
- Negligible baseline noise was observed in the analyte channel when HRAMS selectivity was applied to HCD generated product ion spectra at 17,500 resolving power.
- *Note: RT differed from the final method because an Acquity UPLC HSS T3 1.8 µm, 2.1 x 50 mm column with an analytical flow rate of 500 µL/min was used.*

Method Overview

Sample Extraction

- The triple quadrupole method was originally validated in rat plasma using a hydrophilic-lipophilic-balanced (HLB) solid-phase extraction (LLOQ = 0.25 ng/mL). Background interferences prevented reduction of the LLOQ to levels needed for rabbit plasma. The method was modified to use double liquid-liquid extraction with MTBE to improve recovery.

Scan Rate vs Chromatography

- Orbitrap transient times as a function of required resolving power are displayed below.
- The validated UHPLC triple quadrupole method exhibited a baseline peak width of ~3 sec.
- At low analyte concentrations on the Q Exactive, the baseline peak width was decreased due to limits of detection at the shoulders of the peak elution profile.
- To facilitate adequate peak sampling on the Q Exactive, chromatography was modified to generate a baseline peak width of ~6 sec for mid/high analyte concentrations by reducing flow rate and changing column particle size from 1.7 to 2.5 µm.

Q Exactive Transient Times for Various Resolution Settings

Resolving Power at *m/z* 200	Approximate Scan Speed (Hz)	Approximate Scan Time (ms)	Transient Length (ms)	Suggested Maximum Injection Time (ms)
17,500	13	77	64	50
35,000	7	145	128	110
70,000	3	290	256	240
140,000	1.5	580	512	500

- The approximate scan time was matched with the best resolution for analysis based on obtaining at least 10 scans across the chromatographic peak.
- The maximum injection time was matched to the orbitrap transient length to maximize sensitivity and minimize duty cycle.

Method Development Challenges

Mobile Phase Considerations

- The validated rat plasma method used a basic mobile phase (0.1% ammonium hydroxide/acetonitrile). Improved sensitivity was achieved using 0.5% ammonium hydroxide/methanol.
- Sodium adducts were observed to contribute to reduced analyte response.
- Previous in-house data showed methanol solvent in glass reservoirs contains high levels of sodium. We now routinely use mobile phase solvents shipped in NOWPak (HDPE/PTFE lined).
- Additionally, use of Teflon mobile phase reservoirs reduces sodium adduction. These are used for some sensitive methods in-house.

Mass Spectrometric Detection

- The Q Exactive enabled very high resolution mass detection, up to 140,000 resolving power. This is usually sufficient to resolve most isobaric interferences seen under unit mass resolution conditions.
- Targeted MSX was evaluated but was not found to sufficiently reduce background interference using 3 millimass unit (mmu) processing.
- HCD (MS2) fragmentation provided added selectivity, enabling low detection limits.

Method

Sample Preparation

1. Wash a 2-mL round 96-well plate by adding 500 µL of MTBE to remove possible contaminants. Dry plate.
2. Add plasma sample (200 µL) plus SIL IS to the 96-well plate.
3. Add 800 µL of MTBE to each well using the Microlab STAR (Hamilton Company).
4. Mix on hematology mixer for 10 min.
5. Transfer 600 µL of the organic layer to a clean collection plate and evaporate.
6. Repeat Steps 3 and 4 and transfer to same evaporation plate.
7. After evaporation, reconstitute in water/methanol.

Chromatographic Conditions

LC System:	Dionex/UltiMate 3000 RSLC (Thermo Scientific)
Column:	XBridge BEH C_{18} (2.1 x 50 mm; 2.5 µm particle size; Waters)
Column Temperature:	55 °C
Injection Volume:	30 µL
Mobile Phases*:	A: 1000:5 Water (MilliQ)/Ammonium Hydroxide B: Methanol (NOWPak)
Flow Rate:	250 µL/min
Retention Time:	2.80 min

*Teflon mobile phase bottles were used in place of glass mobile phase bottles.

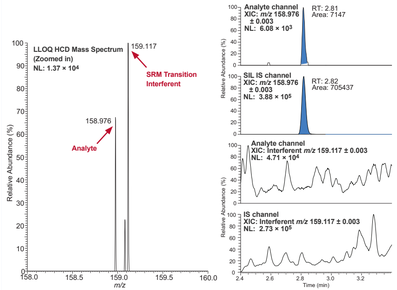
Gradient:	Time (min)	Flow Rate (µL/min)	Mobile Phase B (%)
	0.00	250	45
	3.00	250	95
	4.00	250	95
	5.00	250	45
	6.50	250	45

Mass Spectrometry Conditions

Instrument:	Q Exactive	Auxiliary Gas Pressure:	20 psi
Source:	HESI-II	S-Lens Radio Frequency Level:	50%
Polarity:	Positive	Capillary Temperature:	370 °C
Spray Voltage:	3.8 kV	Heater Temperature:	400 °C
Sweep Cone Pressure:	0 psi		

Acquisition Mode	Targeted MSX (MS1)	HCD (MS2)
Quadrupole Isolation Width	±0.5 *m/z* units	±2.0 *m/z* units
Automatic Gain Control Target	5 × 10⁵	1 × 10⁶
Maximum Injection Time	240 ms	50 ms
Normalized Collision Energy	–	40%
Orbitrap Resolution	140,000	17,500
Accurate Mass Processing (±3 mmu mass tolerance)	Analyte: *m/z* 391.073 SIL IS: *m/z* 398.096	Analyte: *m/z* 158.976 SIL IS: *m/z* 158.976

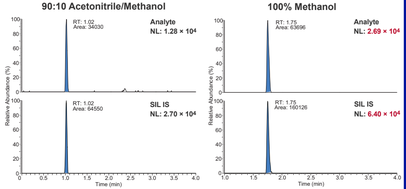
Is the SRM Interference Observed and Resolved in the Q Exactive HCD Data?



- HRAMS enabled the analyte and SIL IS to be separated from the interferent ion to improve selectivity and overall sensitivity.
- The difference between the HCD product ion and interferent ion was only 0.141 Da.
- Unit resolution mass spectrometers were unable to resolve the interferent from the analyte and SIL IS ions.

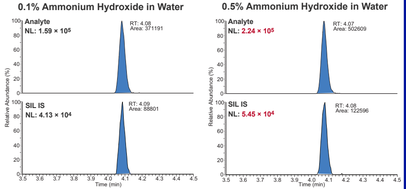
Method Development Changes to Improve Sensitivity

Mobile Phase: Changing Mobile Phase B to 100% Methanol



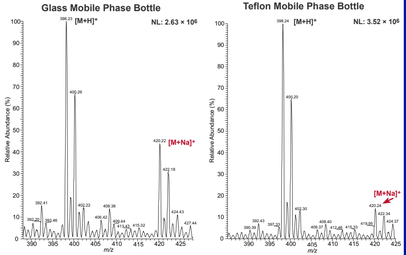
- A 2-fold gain in sensitivity was observed by changing Mobile Phase B to 100% methanol.
- *Note: RT was different than the final assay because an Acquity UPLC HSS T3 1.8 µm, 2.1 x 50 mm column with an analytical flow rate of 500 µL/min was used.*

Mobile Phase: Increasing Percentage of Ammonium Hydroxide in Mobile Phase A



- A 1.5-fold gain in sensitivity was observed by increasing the ammonium hydroxide concentration in Mobile Phase A.
- *Note: RT was different than the final assay because a Waters XBridge BEH C_{18} 2.5 µm, 2.1 x 50 mm column with an analytical flow rate of 100 µL/min was used.*

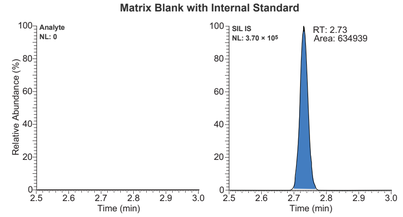
Glass Bottles vs Teflon Bottles as Mobile Phase Reservoirs



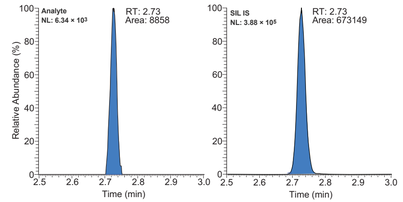
- Sodium adduction was decreased by use of Teflon mobile phase reservoirs for mobile phase solutions.
- A 2-fold gain in sensitivity was observed in UHPLC runs on the Q Exactive.
- *Note: These data were collected on a TSQ Vantage MS equipped with the same HESI-II source in Q1 only mode.*

Results

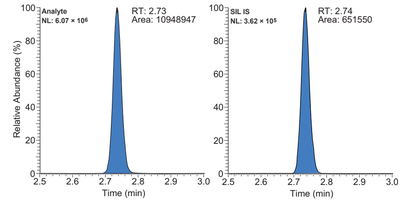
Matrix Blank with Internal Standard



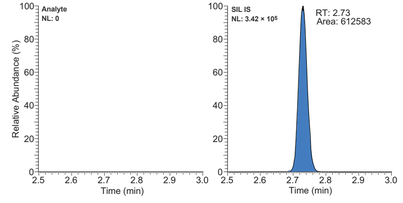
LLOQ (2 pg/mL)



Upper Limit of Quantitation (ULOQ; 2000 pg/mL)

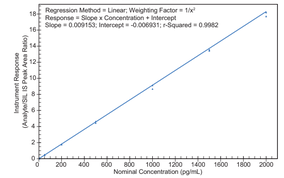


Primary Carryover Blank



Excellent selectivity and negligible carryover were observed with this method on the Q Exactive with HCD.

Standard Curve



Excellent linear dynamic range was observed with this method on the Q Exactive with HCD.

Inter-Day Method Accuracy and Precision

	GSKA Concentration (pg/mL)				
	LLOQ QC	QC1	QC2	QC3	QC4
	2.00	6.00	60.0	800	1600
Mean	2.29	6.41	62.8	799	1570
SD	0.178	0.325	1.88	26.3	61.5
CV (%)	7.8	5.1	3.0	3.3	3.9
RE (%)	14.5	6.8	4.7	-0.1	-1.9
n	18	18	18	18	18

CV (%) = (SD/Mean) × 100
RE (%) = [(Mean − Nominal)/Nominal] × 100

- Quality control (QC) sample statistics indicate good accuracy and precision from three method validation runs across the linear dynamic range of 2.00 to 1600 pg/mL.
- The LLOQ in rabbit plasma was established at 2 pg/mL because adequate precision was not demonstrated at 1 pg/mL (data not shown).

Inter-Lot Accuracy and Precision

		GSKA Concentration (pg/mL)						
	Plasma Lot	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5	Lot 6*	Inter-lot
LLOQ 2.00 pg/mL	Replicate 1	2.01	1.80	2.11	1.94	2.55	1.65	
	Replicate 2	2.06	1.66	1.86	1.46	2.12	1.69	
	Replicate 3	1.79	2.33	2.22	2.13	2.00	1.57	
	Mean	1.95	1.93	2.06	1.84	2.22	1.64	1.94
	SD	0.144	0.353	0.184	0.345	0.289	0.0611	0.283
	CV (%)	7.4	18.3	8.9	18.8	13.0	3.7	14.6
	RE (%)	-2.5	-3.5	3.0	-8.0	11.0	-18.0	-3.0
	n	3	3	3	3	3	3	18
ULOQ 2000 pg/mL	Replicate 1	2290	1960	1930	1900	1930	1980	
	Replicate 2	2290	1930	1880	1970	1890	1900	
	Replicate 3	2370	1960	1900	1990	1900	1920	
	Mean	2320	1950	1900	1950	1910	1960	2000
	SD	46.2	17.3	25.2	47.3	20.8	32.1	151
	CV (%)	2.0	0.9	1.3	2.4	1.1	1.6	7.6
	RE (%)	16.0	-2.5	-5.0	-2.5	-4.5	-2.0	0.0
	n	3	3	3	3	3	3	18

*Plasma lot with 2% hemolysis.

- Adequate inter-lot accuracy and precision and inter-lot selectivity were observed when processing the HCD data with a ±0.003 Da XIC mass tolerance.
- QC sample statistics indicate good accuracy and precision from six individual lots of rabbit plasma, including one lot with 2% hemolysis.
- In addition, there were no chromatographic interferences observed among the same six individual lots of rabbit plasma, including one lot with 2% hemolysis.

Conclusions

- Sensitivity was a challenge in the development of the GSKA method in rabbit plasma due to isobaric interference and sodium adduction.
- Isobaric interference was not resolvable by MS1 high-resolution mass spectrometry alone. HCD (MS2) fragmentation was required for selectivity and sensitivity.
- Sodium adduction was minimized by use of non-glass mobile phase solvent reservoirs.
- The method for GSKA in rabbit plasma was successfully developed, validated, and used in regulated preclinical sample analysis.

Acknowledgment: Suzanne Spencer for poster creation