Comparison of UPLC-Triple Quadrupole MS and UPLC-High Resolution MS for Human Microdosing Assays With Pico-gram per Milliliter Quantification Limit

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Abstract

Introduction: Support of human microdosing studies requires ultrasensitive assays to monitor clinical pharmacokinetics profiles of study compounds at microgram dose levels. The major challenge in developing such assays is to differentiate trace amounts of compound signal from large matrix background. To explore the potential advantages of applying HRMS to optimize assay sensitivity by resolving isobaric matrix interferences, 2 compounds (**C-X** and **C-Y**) were tested and compared using traditional tandem triple quadrupole mass spectrometry (QQQ) and high-resolution mass spectrometry (HRMS).

Method: Compounds **C-X** and **C-Y** were spiked into human EDTA plasma separately and extracted with Agilent PLEXA solid-phase extraction (SPE) plates. The resulting extract was chromatographically separated on a Waters Shield RP18 (50 x 2 mm, 1.7 µm) column with Waters Acquity UPLC system and quantitated using either a Sciex 6500 QQQ with Turboionspray (TIS) under MRM mode or a Waters Xevo G2-XS QToF HRMS with ESI using ToF MRM with target enhancement in sensitivity mode.

Results: Regression of 5 standard curves extracted from 5 different lots of human plasma with a dynamic range of 2-2,000 pg/mL provided comparable precision (<10%) and accuracy (mean % bias <11%) for **C-X**, with a lower limit of quantification (LLOQ) at 2 pg/mL, on both QQQ and HRMS. Similar results were achieved for **C-Y** with QQQ; however, the LLOQ observed on the Xevo G2-XS was approximately 5-fold higher (10 pg/mL).

Conclusion/Novel Aspects: Increasing numbers of reports have shown that the use of HRMS could improve assay sensitivity.[1,2] Our evaluation results suggest that the LLOQ obtained on HRMS appears to be compound dependent. After SPE sample cleanup, the Xevo G2-XS provided comparable sensitivity for **C-X**, but less sensitivity for **C-Y** by a factor of 5, compared to the data obtained using the Sciex 6500 QQQ. The extent of sample cleanup may play an important role in this comparison. The mass resolving power of HRMS could potentially be more advantageous when applied to a relatively cruder plasma extract (eg, through protein precipitation or liquid-liquid extraction), which may minimize the method development effort in terms of sample preparation.

Introduction

- A microdosing study was planned to compare the PK behavior of the selected compounds in humans at a dose level of 100 µg. The results of this microdosing study were anticipated to be used to inform on human clearance and advance one of the study compounds to preclinical candidacy
- Quantification of all study compounds required ultrasensitive assays with 2 pg/mL quantification limit
- The triple quadrupole MS (QQQ) is currently the primary tool for quantitative bioanalysis due to high sensitivity
- QTOF HRMS is mainly used for qualitative workflow; recently, interest in using HRMS to address sensitivity issues in quantitative analysis has emerged
- A comparison between QQQ and HRMS was conducted using two of the microdosing compounds (**C-X** and **C-Y**) that had relatively low MS response on QQQ

Evaluation plan

- Selected compounds: **C-X** and **C-Y** from microdosing study
- Use the same extraction procedures and UPLC conditions
- Compare the data from different MS platforms:
  - QQQ: Sciex API 6500 TISP, positive mode
  - QToF HRMS: Waters Xevo G2-XS

Sample Preparation Challenges

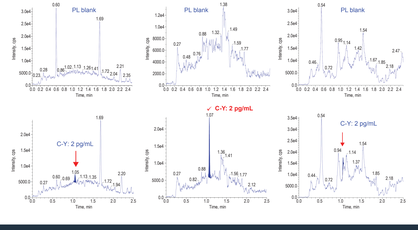
Liquid-liquid extraction (LLE)

- Optimized LLE conditions under different pH (pH 5 vs 10) with different extraction solvent (MtBE vs EtOAc)
- Result: Very low signal-to-noise ratio at 2 pg/mL for both **C-X** and **C-Y** (**Figure 1a**)

Solid-phase extraction (SPE) – sorbent selection

- Waters uElution plate (HLB and MAX) for 400-µL sample – high background from HLB extract and significant loss from MAX
- Agilent Bond Elut PLEXA, PAX, and PCX for 1-mL plasma sample
  - PLEXA gave reasonable recovery but high matrix effect
  - PAX gave good recovery with neat solution but very low (~10%) recovery with plasma sample
  - PCX – significant loss during loading step
- PLEXA 10 mg vs 30 mg (**Figure 1b and 1c**)
  - In general, 30 mg is recommended for handling 1 mL plasma
  - However, 30 mg PLEXA gave significantly high background and low signal-to-noise ratio
  - Instead, 10 mg PLEXA was selected as the final choice for **C-X** and **C-Y**
- Optimize each step of PLEXA extraction, including conditioning, loading, washing, and eluting

Figure 1. Chromatograms from different extraction methods (C-Y, LLOQ at 2 pg/mL)



Final Assay Procedure for C-X and C-Y

SPE with Agilent Bond Elut PLEXA (2 mL, 10 mg) plate and UPLC conditions:

- Plasma volume: 1 mL
- SPE – automated on Tomtec (manual vacuum control):
  - Mixing PL with acid solution using Tomtec (no rotation mixing)
  - Conditioning with ACN (0.5 mL) and then water (0.5 mL twice – for consistent loading)
  - Loading under acidic condition
  - Wash with basic washing solvent (0.5 mL twice – for cleaner extract)
  - Elute under acid in acetonitrile, dry under N_2
- Reconstitution: 50 µL 20% ACN with 0.1% FA
- Injection volume: 10 µL
- UPLC conditions (gradient of mobile phase A and B on Acquity):
  - Solvent A: 0.1% FA in water
  - Solvent B: 0.1% FA in acetonitrile

Comparison Between QQQ and HRMS

QQQ: C-X and C-Y

- Specificity:
  - Double blank – no peak in **C-X** or **C-Y** channels at the retention time of **C-X** or **C-Y** (**Figures 2a and 3a**)
- Good regression based on the 5 curves from 5 lots of plasma, including hyperlipidemic and hemolyzed lots (**Table 1**)
  - Accuracy: 97.3%-110.2% and 97.3%-109.8% for **C-X** and **C-Y**, respectively
  - Precision (%CV): <9.1% and <5% for **C-X** and **C-Y**, respectively
  - Good signal-to-noise ratio at LLOQ (**Figures 2b and 3b**)
  - No significant carryover after the ULOQ injection (**Figures 2d and 3d**)
- Used sum of 2 MRMs for **C-Y** to get better accuracy/precision at LLOQ

Figure 2. Representative chromatograms of C-X on QQQ

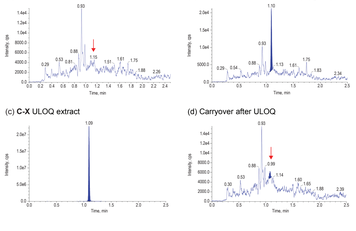
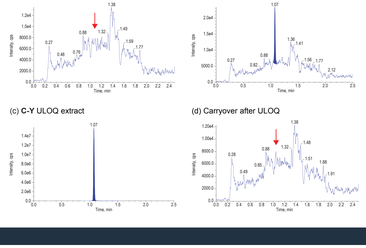


Figure 3. Representative chromatograms of C-Y on QQQ



Comparison Between QQQ and HRMS (cont'd)

Table 1. Precision and accuracy of C-X and C-Y from 5 different lots of human plasma (QQQ)

Sample ID	Nominal Conc. (pg/mL)	C-X (n=5)		C-Y (n=5)	
		Accuracy (%)	% CV	Accuracy (%)	% CV
STD 1	2	100.8	9.12	101.0	4.97
STD 2	4	99.6	5.19	99.4	2.90
STD 3	10	97.8	3.06	97.3	2.98
STD 4	40	98.0	5.32	96.9	1.28
STD 5	200	97.3	2.66	98.9	2.90
STD 6	1,000	98.0	5.54	98.4	2.65
STD 7	1,600	110.2	4.52	109.8	0.64
STD 8	2,000	98.3	4.84	97.8	2.28

(Weight: $1/x^2$ linear regression)

Recovery and matrix effect on QQQ

- **C-X**:
  - Recovery ranged from 69% to 81% for both analyte and ISTD
  - Absolute matrix effect ranged from 57% to 60% (significant ME but consistent across 5 different lots of plasma)
  - ME Factor based on peak area ratio ranged from 98% to 102%
- **C-Y**:
  - Recovery ranged from 68% to 71% for both analyte and ISTD
  - Absolute matrix effect ranged from 66% to 70% (significant ME but consistent across 5 different lots of plasma)
  - ME Factor based on peak area ratio ranged from 98% to 102%

HRMS: C-X and C-Y

- Compound behavior on Xevo G2-XS:
  - Similar to QQQ, 2 significant product ions were observed for **C-Y** (**Figure 4**)
  - On Xevo G2-XS, TOF-MRM using sensitivity mode with target enhancement (TE) provided the best signal-to-noise ratios
    - TE improved signal by about 10-fold (**Figure 5**)
    - Sensitivity mode with TE gave the best signal-to-noise ratio for full-scan ToF MS (**Figure 6a**)
    - Sensitivity mode with TE (continuum) provided additional sensitivity to ToF MRM (**Figure 6b**)
- Specificity:
  - Double blank – no peak in **C-X** or **C-Y** channels at the retention time of **C-X** or **C-Y** (**Figures 7a and 8a**); specificity was also achieved in the SIL-ISTD channels
- The standard curves regression from 5 lots of plasma, including hyperlipidemic and hemolyzed lots, are summarized in **Table 2**
  - Similar LLOQ (2 pg/mL) was achieved for **C-X**, while about 5x worse sensitivity (LLOQ ~10 pg/mL) was observed for **C-Y** on HRMS
  - The S/N ratios at their corresponding LLOQs were reasonable (**Figures 7b and 8b**)

Figure 4. Product scan of C-X and C-Y on Xevo G2-XS

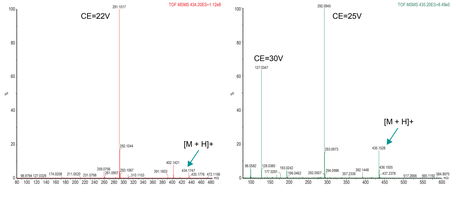


Figure 5. Effect of target enhancement for the C-X product ion on Xevo G2-XS

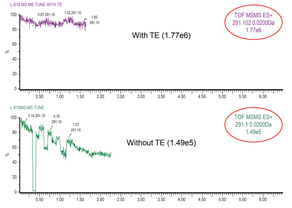


Figure 6. Effect of resolution and sensitivity modes on S/N for C-X using Xevo G2-XS

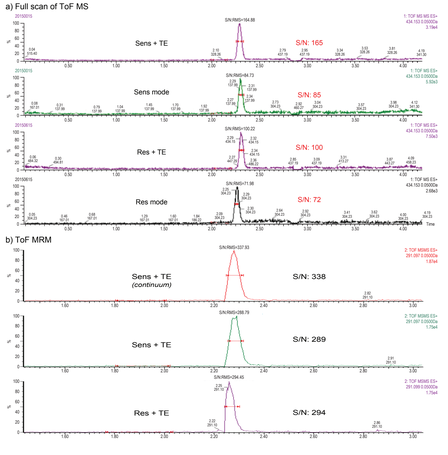


Figure 7. Representative chromatograms of C-X using HRMS

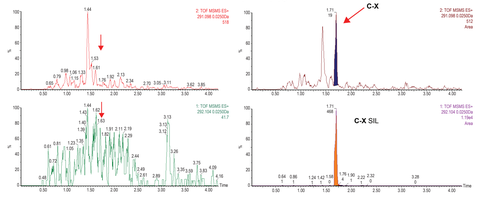


Figure 8. Representative chromatograms of C-Y using HRMS

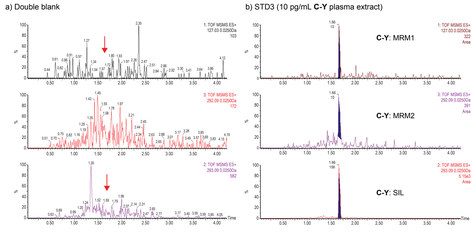


Table 2. Precision and accuracy of C-X and C-Y from 5 different lots of human plasma (HRMS)

Sample ID	Nominal Conc. (pg/mL)	C-X (n=5)		C-Y (MRM1) (n=5)		C-Y (MRM2) (n=5)	
		Accuracy (%)	% CV	Accuracy (%)	% CV	Accuracy (%)	% CV
STD 1	2	96.9	9.1	-65.5	58.2	-129.3	43.6
STD 2	4	102.6	9.5	41.8	66.0	-25.4	28.4
STD 3	10	108.4	5.2	57.6	20.1	85.2	19.3
STD 4	40	101.5	1.1	98.2	6.5	98.4	14.5
STD 5	200	107.8	5.3	109.6	8.9	108.2	5.8
STD 6	1,000	89.6	3.6	103.4	6.6	104.3	7.7
STD 7	1,600	96.9	2.4	100.2	6.4	100.5	5.6
STD 8	2,000	96.4	3.4	88.7	1.9	88.6	2.1

(Weight: $1/x^2$ linear regression)

Conclusion

- The LLOQ observed using HRMS was compound dependent
  - Compared to the data obtained using Sciex 6500 QQQ, Waters Xevo G2-XS provided comparable LLOQ for **C-X** but less sensitivity for **C-Y** by a factor of 5
- Future directions:
  - Summing multiple product ions could be explored on the G2-XS Q-ToF, which may provide additional sensitivity for **C-Y**
  - HRMS may be more beneficial when applied to the relatively cruder plasma extract (eg, through protein precipitation or LLE). HRMS could provide additional resolving power (improved S/N) if specificity issue is encountered using QQQ

References

1. Evens C. When you need a sensitive and selective method, the answer could be HR-MS?! The 9th Workshop on Recent Issues in Bioanalysis (WRIB), Miami, FL, 2015.
2. Sun L, Bateman K, Alelyunas Y, Wrona M. Reducing matrix interference using ionKey/HRMS for the analysis of raltegravir in human plasma. The 8th European Bioanalytical Forum (EBF), Barcelona, Spain, 2015.