

## INTRODUCTION

- Sildenafil has been used for treatment of pulmonary arterial hypertension, erectile dysfunction, and as a potential treatment for congenital diaphragmatic hernia.
- Sildenafil and its metabolite have been shown to selectively inhibit phosphodiesterase type 5, so quantifying them both simultaneously is important.
- Previously reported LC-MS/MS methods to quantify sildenafil and N-desmethyl sildenafil require large sample volumes, long runtimes, or complex sample preparation.
- A simplified UPLC-MS/MS assay was developed for the quantitative determination of sildenafil and N-desmethyl sildenafil in serum, amniotic fluid and tissue (brain, eye, kidney, lung, and forearm) samples.
- Sildenafil and its metabolite concentration were measured in mice and rabbit samples to demonstrate the validity of the method.

## OBJECTIVE

- To develop and validate a method for the quantification of sildenafil and its metabolite in serum, tissues, and amniotic fluid from mice and rabbits.

## METHODS

- Serum, tissue, and amniotic fluid samples (50µL) underwent protein precipitation with a methanol-internal standard solution.
- Standards and quality controls were prepared in stripped serum (serum/tissue) or 1% BSA in PBS (amniotic fluid).
- The separation of the samples was performed using an Acquity BEH C18 (2.1 mm x 100 mm, 1.7 µm) column with a BEH C18 1.7 µm VanGuard Pre-Column.
- The mobile phase consisted of water with 0.1% formic acid (solvent A) and methanol (solvent B).
- A gradient elution at a flow rate of 0.3 mL/min was run on an Acquity UPLC I-class (Waters) with a total run time of 3 minutes.
- Analytes were detected in positive ion mode with selected reaction monitoring (SRM) using a heated electrospray ionization (HESI) source for ionization on a triple quadrupole mass spectrometer (Thermo Scientific).

Figure 1. The chemical structures of the analyte and internal standard.

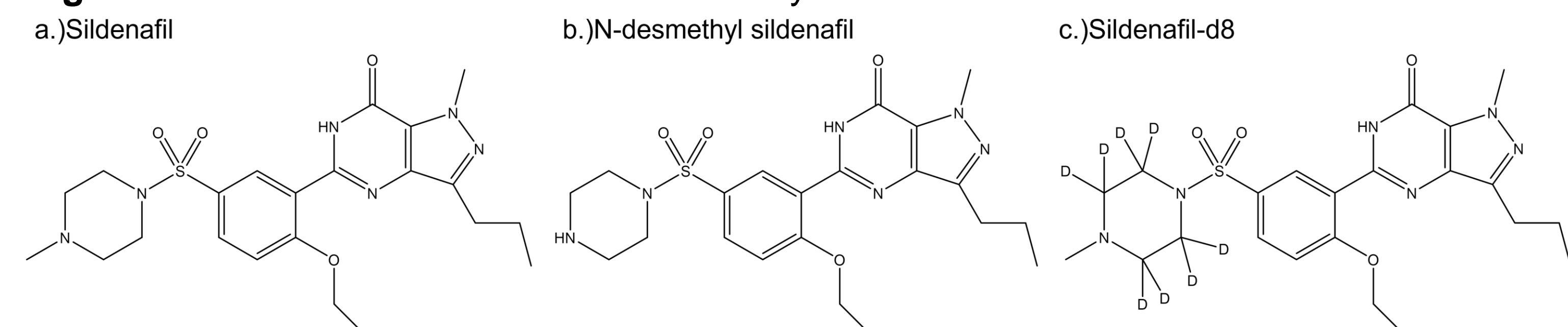


Table 1. SRM parameters for the analyte and internal standard.

Analyte	Precursor Ion (m/z)	Product Ion (m/z)	Collision Energy (V)	RF Lens (V)
Sildenafil	475.2	283.1	40	190
Sildenafil-d8	483.3	283.1	39	122
N-desmethyl sildenafil	461.0	283.1	36	159

## RESULTS

Table 2. Results of the assay validation including LLOQ and linear range. The mean slopes, y-intercepts, and correlation coefficient for both analytes are shown.

	Sildenafil		N-desmethyl Sildenafil	
	Serum and Tissue	Amniotic fluid	Serum and Tissue	Amniotic fluid
LLOQ (ng/mL)	0.5	0.5	0.5	0.5
Linear Range (ng/mL)	0.5-1000	0.5-1000	0.5-1000	0.5-1000
Slope (mean)	0.0025	0.0025	0.0080	0.0086
Intercept (mean)	-0.0002	-0.0004	-0.00018	-0.0019
Correlation Coefficient (mean)	0.9977	0.9980	0.9967	0.9964

Table 3. Intra- and inter-day accuracy (%deviation) and precision (%CV) for LLOQ and QCs.

	Level	Nominal Concentration	Intra-day <sup>a</sup>				Inter-day <sup>b</sup>			
			% Deviation		% CV		% Deviation		% CV	
			Sildenafil	N-desmethyl	Sildenafil	N-desmethyl	Sildenafil	N-desmethyl	Sildenafil	N-desmethyl
Serum/Tissue (ng/mL)	LLOQ	0.5	8.6	12.5	3.6	3.4	9.4	9.7	5.5	5.4
	LQC	1.5	-3.3	-3.5	6.4	5.8	-2.2	-1.3	7.4	7.4
	MQC	400	3.9	-4.7	2.7	2.8	2.8	-3.0	4.3	5.8
	HQC	800	2.3	5.0	1.7	3.0	1.4	4.4	4.0	5.0
Amniotic Fluid (ng/mL)	LLOQ	0.5	11.4	12.5	3.3	3.4	9.2	9.7	5.2	5.4
	LQC	1.5	5.4	-3.5	5.7	5.8	3.2	-1.3	7.3	7.4
	MQC	400	-3.4	-4.7	4.0	2.8	-2.4	-3.0	6.0	5.8
	HQC	800	-3.8	5.0	2.6	3.0	3.8	4.4	4.9	5.0

<sup>a</sup>12 replicates for QCs.

<sup>b</sup>24 replicates for QCs.

Table 4. Recovery and matrix effect of sildenafil and n-desmethyl sildenafil from serum, tissue, and amniotic fluid (n = 3).

	QC Level	Nominal Concentration	Recovery (% mean)		Matrix Effect (% mean)	
			Sildenafil	N-desmethyl Sildenafil	Sildenafil	N-desmethyl Sildenafil
			Serum/Tissue (ng/mL)	LQC	100.24	100.58
	MQC	98.43	100.38	108.28	104.90	
	HQC	95.93	101.42	106.45	98.28	
Amniotic Fluid (ng/mL)	LQC	98.95	102.05	99.34	104.44	
	MQC	97.59	100.34	97.48	94.39	
	HQC	100.74	104.66	101.33	98.25	

Table 5. Stability of sildenafil and n-desmethyl sildenafil in serum, tissue and amniotic fluid (n = 3).

	QC Level	Nominal Concentration	Bench Top Stability (RT, after 4 h)		Autosampler Stability (10°C, after 72 h)		Freeze/Thaw Stability (-80°C, after 3 cycles)	
			Sildenafil	N-desmethyl Sildenafil	Sildenafil	N-desmethyl Sildenafil	Sildenafil	N-desmethyl Sildenafil
			Serum/tissue (ng/mL)	LQC	98.7	102.2	104.1	106.9
	HQC	101.3	101.0	105.1	105.2	96.1	98.3	
Amniotic fluid (ng/mL)	LQC	98.7	94.5	99.5	99.5	100.7	92.1	
	HQC	102.2	105.7	105.7	101.0	103.4	95.7	

## RESULTS

Figure 2. EICs for a) n-desmethyl sildenafil, b) sildenafil, and c) sildenafil-d8 for the LLOQ, low, middle, and high quality control samples in stripped human serum.

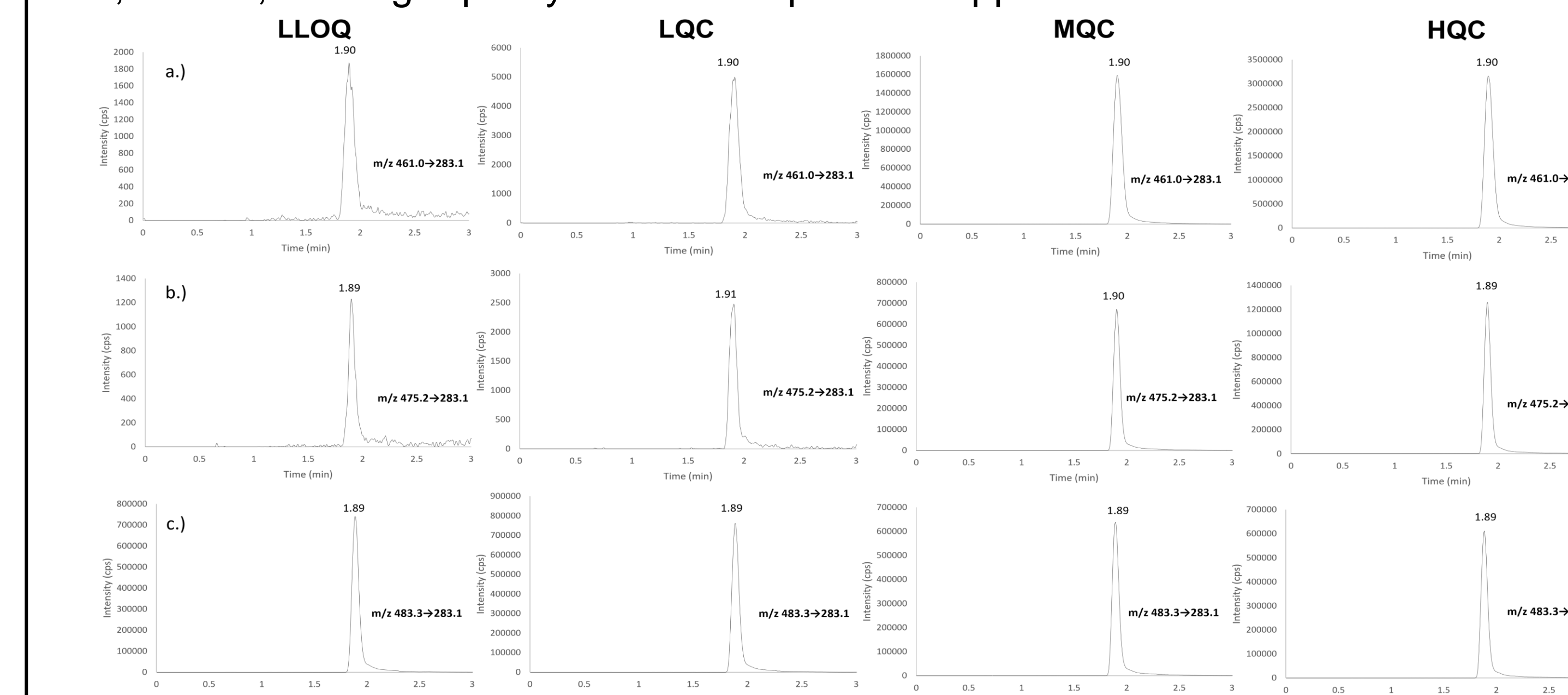
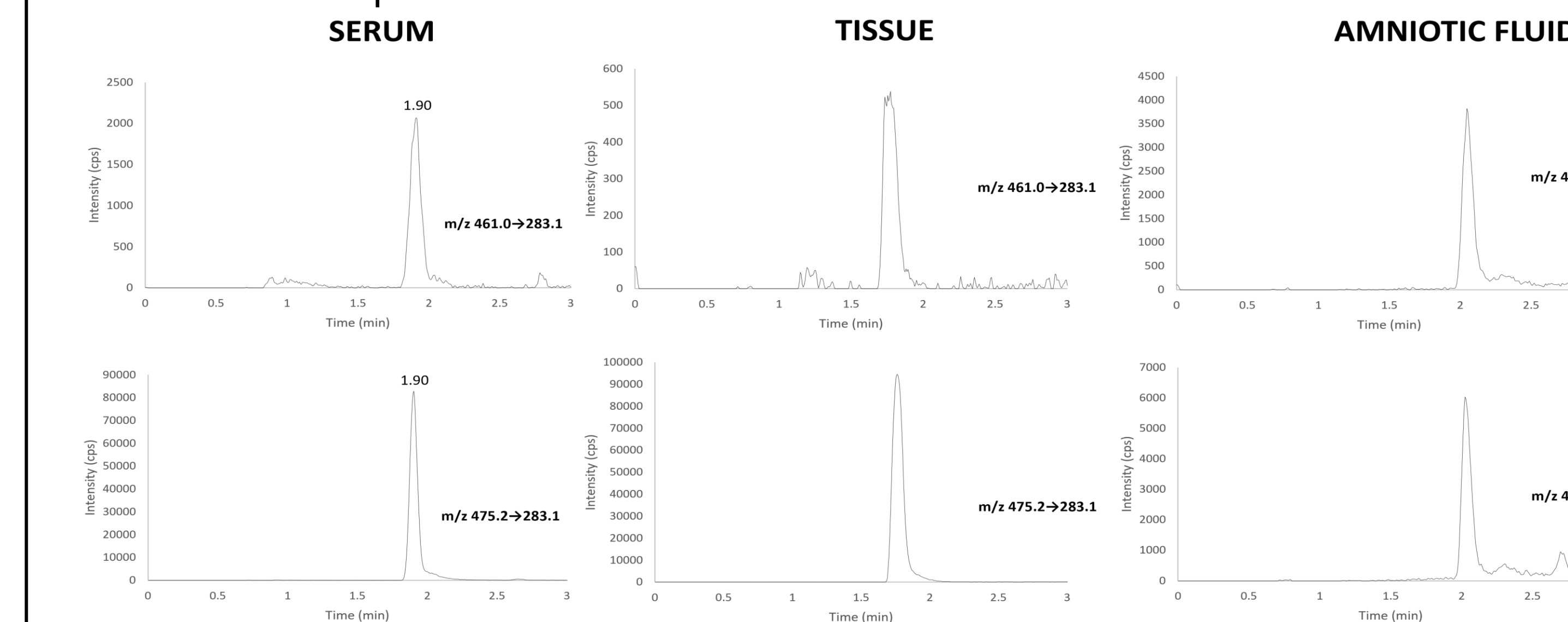


Figure 3. EICs for a) n-desmethyl sildenafil and b) sildenafil from mouse serum, tissue, and amniotic fluid samples.



## CONCLUSIONS

- A sensitive, simple, and high throughput UPLC-MS/MS assay for the quantification of sildenafil and n-desmethyl sildenafil in serum, tissue, and amniotic fluid samples was developed and comprehensively validated according to FDA guidelines.
- The assay has several advantages over existing assays including small sample volume requirements, minimal sample preparation, high-throughput capacity, and great accuracy and precision.
- The method was applied to measure sildenafil and its metabolite in serum, tissue, and amniotic fluid in mice and rabbits for a clinical study to show proof of concept.

## ACKNOWLEDGMENT

Supported by the NIH grant S10OD028540 titled "Small Molecule Biomarker Core: TSQ Altis LC-MS/MS"