Enhanced Metabolite Characterization of Therapeutic siRNAs Using Data Independent, Sequential Windowed Acquisition of all THeoretical (SWATH) Ions, LC-MS/HRMS Methods

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INTRODUCTION

Characterizing metabolites of drug-related small interfering RNAs (siRNAs) is essential for pharmacokinetic and safety assessment but remains challenging due to complex biotransformation pathways and the presence of isomeric species with multiple charge states. Traditional data-dependent acquisition requires prior knowledge of precursor ions to trigger MS/MS events, which can limit detection of unknown or low-abundance metabolites. Data-independent acquisition using Sequential Windowed Acquisition of all THeoretical (SWATH) Ions enables untargeted MS/MS collection across all ions without prior metabolite information, improving detection sensitivity. This study shows the application of SWATH acquisition for characterizing biotransformation metabolites of siRNA therapeutics.

METHODS

Oligonucleotide related metabolites were generated through *in vitro* incubations in rat, monkey, and human liver S9 fractions using commercially available siRNAs Givosiran®, Inclisiran®, and Patisiran® as model compounds. Samples were extracted by solid-phase extraction (SPE) and analyzed by Shimadzu UHPLC coupled to a SCIEX TripleTOF® 6600 in negative ESI mode. Chromatographic separation using an Acquity BEH C18, 1.7 µm (Waters) column with the ion-pairing mobile phases A (H_2O:DIPA:HFIP) and B (H_2O:MeOH:DIPA:HFIP). SWATH enabled comprehensive untargeted MS/MS characterization of precursor ions with a window of 55 Da. Data were processed using SCIEX OS Explorer, Analytics, and Molecule Profiler. For structural elucidation of isomeric metabolites, targeted LC-MS/HRMS methods were applied.

Interpretation for Givosiran®, Inclisiran®, and Patisiran®, and L96 Terminal Group

- Givosiran (Givlaari®) (sodium salt; MW 17,246 Da)

  5' mUsfAsfAfGmAfUmGfAmGfAmCfAmCfUmCfUmUfUmCfUmGsmGsmU 3'

  3' L96-mAmUmUmCmUmAfCmUfCmUfGmUfGmAfGmAmAmGsmAsmC 5'

- Inclisiran (Leqvio®) (sodium salt; MW 17,285 Da)

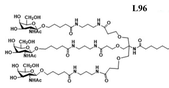
  5' mAsfCsmAfAfAfAmGfCmAfAmAfAmCfAmGfGmUfCmUmAmGsmAsmA 3'

  3' L96-mUmGmUmUmUmUmCmGmUmUdTmUfGmUfCmCmAmGmAsmUsmC 5'

- Patisiran (Onpattro®) (LNP formulation; sodium salt; MW 14,304 Da)

  5' rArUrGrGrArAmUrArCrUrCrUrUrGrGrUmUrArCdTdT 3'

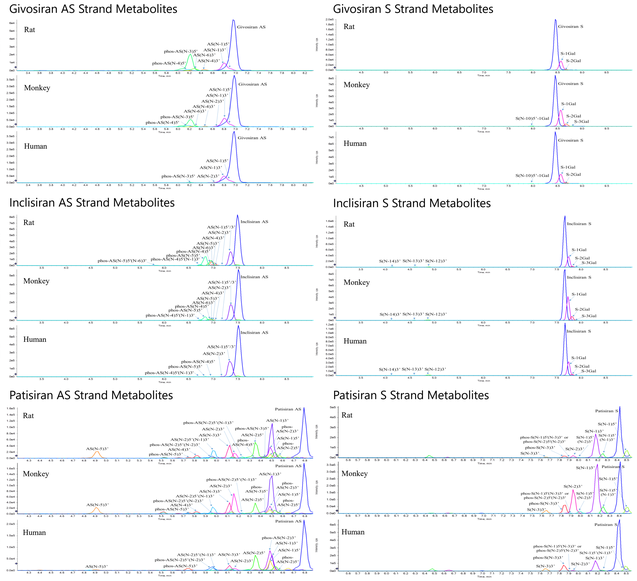
  3' dTdTmUrAmCmCmUmUrAmUrGrArGrArAmCmCrArAmUrG 5'



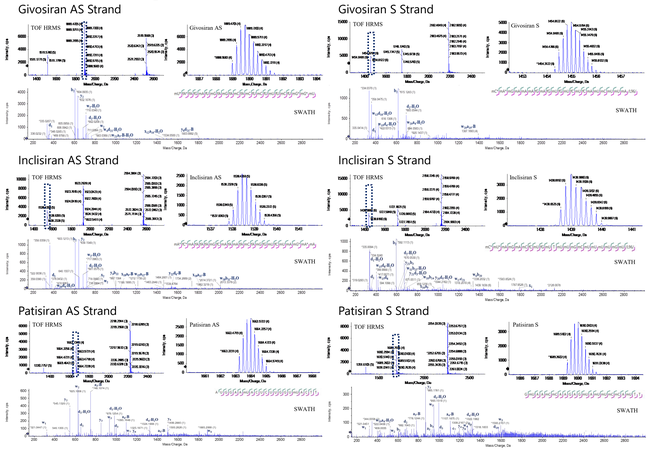
All phosphate linkage except those specified. The above two siRNAs in which the sense (or passenger) strand is covalently linked to three GalNAc moieties facilitating the delivery via the guide strand, also containing two 3'-overhang ribonucleotides connected through phosphorothioate linkages.
T = Thymine, A = Adenine, C = Cytosine, G = Guanine, U = Uracil; d = 2'- H (Deoxy-); f = 2'-Fluoro; m = 2'-O-Methyl; s = phosphorothioate.

RESULTS

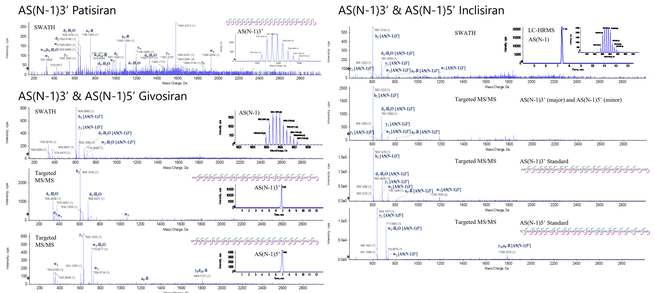
UHPLC Separation of Givosiran®, Inclisiran®, and Patisiran® Metabolites in Liver S9 Fractions Over 72 Hours Incubation



Characterization of Givosiran®, Inclisiran®, and Patisiran® in Liver S9 Fractions Using Q-TOF-HRMS Combined with SWATH MS/MS



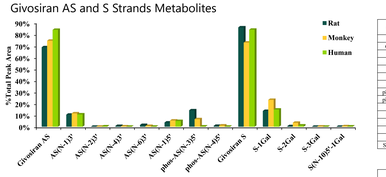
Representative Metabolite Characterization in Liver S9 Fractions Using Q-TOF-HRMS, SWATH, and Targeted MS/MS



- Samples were screened for potential metabolites using UHPLC-Q-TOF-HRMS full-scan, followed by SWATH acquisition to collect comprehensive MS/MS datasets, enabling retrospective extraction of fragment ion profiles without prior knowledge of metabolites or additional sample injections.
- Untargeted SWATH MS/MS profiling generated a greater number of metabolite fragment features that enhanced confidence in metabolite assignments derived from TOF-HRMS survey scans and demonstrated improved sensitivity for detecting low-abundance metabolites by fragmenting entire isotopic ion clusters.
- Follow-up targeted MS/MS enabled differentiation of metabolites sharing identical mass — like 5'- and 3'-truncated (N-1). Through optimized transitions and collision energies, these isomeric metabolites could be selectively isolated and fragmented. Diagnostic fragment ions enabled confident structural assignment.

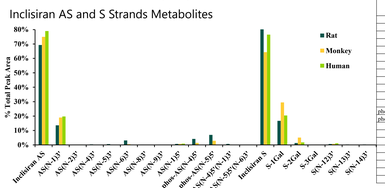
Semiquantitation of Metabolites in Liver S9 Fractions

Givosiran AS and S Strands Metabolites



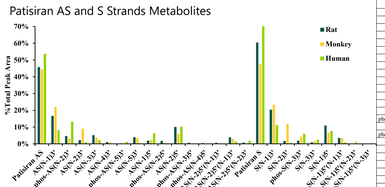
Component	% Total Peak Area		
	Rat	Monkey	Human
AS Strand			
Givosiran AS	68.64%	74.29%	83.67%
ASN-1)3'	10.46%	11.56%	10.67%
ASN-2)3'	ND	0.10%	0.31%
ASN-4)3'	0.71%	0.32%	0.03%
ASN-6)3'	1.54%	0.70%	0.06%
ASN-1)5'	3.65%	5.38%	4.98%
phos-ASN-3)5'	14.20%	6.54%	0.25%
phos-ASN-4)5'	0.80%	1.11%	0.05%
S Strand			
Givosiran S	85.81%	72.70%	83.79%
S-1Gal	13.65%	23.20%	14.86%
S-2Gal	0.54%	3.45%	1.12%
S-3Gal	ND	0.21%	ND
SGN-10)5'-1Gal	ND	0.43%	0.23%

Inclisiran AS and S Strands Metabolites



Component	% Total Peak Area		
	Rat	Monkey	Human
AS Strand			
Inclisiran AS	69.35%	74.98%	79.03%
ASN-1)3'	13.59%	16.98%	19.71%
ASN-2)3'	0.10%	0.15%	0.23%
ASN-4)3'	0.38%	0.05%	ND
ASN-5)3'	0.54%	0.07%	ND
ASN-6)3'	3.08%	0.49%	ND
ASN-8)3'	0.16%	0.02%	ND
ASN-9)3'	0.20%	0.02%	ND
ASN-1)5'	0.69%	0.71%	0.89%
phos-ASN-4)5'	4.16%	1.37%	0.07%
phos-ASN-5)5'	6.95%	2.88%	0.07%
phos-ASN-8)5'(N-1)3'	0.65%	0.28%	0.01%
phos-ASN-5)5'(N-4)3'	0.15%	ND	ND
S Strand			
Inclisiran S	80.71%	84.37%	76.51%
S-1Gal	16.72%	14.49%	20.44%
S-2Gal	1.29%	1.08%	1.72%
S-3Gal	0.04%	0.10%	0.03%
SGN-1)3'	0.62%	0.66%	1.09%
SGN-1)3'	0.16%	0.16%	0.05%
SGN-1)4'5'	0.34%	0.03%	0.16%

Patisiran AS and S Strands Metabolites



Component	% Total Peak Area		
	Rat	Monkey	Human
AS Strand			
Patisiran AS	45.60%	44.49%	53.70%
ASN-1)3'	16.70%	21.88%	8.21%
phos-ASN-2)3'	4.70%	3.14%	13.17%
ASN-2)3'	2.21%	9.04%	1.05%
ASN-3)3'	5.18%	3.74%	2.22%
ASN-4)3'	1.07%	0.84%	0.08%
phos-ASN-5)3'	0.27%	0.41%	1.20%
ASN-5)3'	3.90%	3.48%	0.59%
ASN-1)5'	1.90%	2.51%	6.20%
phos-ASN-2)5'	1.81%	0.65%	0.25%
ASN-2)5'	10.07%	5.83%	10.27%
phos-ASN-3)5'	0.65%	0.20%	0.12%
phos-ASN-4)5'	0.40%	0.18%	0.04%
phos-ASN-2)5'(N-1)3'	0.65%	0.37%	ND
ASN-2)5'(N-1)3'	3.90%	2.97%	1.23%
phos-ASN-2)5'(N-2)3'	0.70%	0.28%	1.86%
S Strand			
Patisiran S	60.39%	47.48%	70.62%
S-1Gal	20.37%	27.51%	11.26%
S-2Gal	1.69%	11.89%	0.63%
phos-SGN-3)5'	1.92%	4.23%	6.03%
SGN-3)3'	0.89%	1.62%	2.51%
SGN-1)5'	10.89%	6.63%	7.65%
SGN-1)5'(N-1)3'	3.48%	3.35%	0.95%
SGN-1)5'(N-2)3'	0.25%	1.29%	ND
phos-SGN-1)5'(N-3)3'	0.14%	0.19%	0.36%

CONCLUSIONS

- Integrated workflow combining UHPLC-Q-TOF-HRMS and untargeted SWATH screening with targeted MS/MS confirmation to enhance siRNA sensitivity, metabolite coverage, and structural confidence.
- There were no disproportional or unique human metabolites for three siRNAs in liver S9 fractions.
- Limited to moderate metabolisms were observed in both AS and S strands for three siRNAs.
- Observed major metabolites were shorter nucleotides (N-x) and loss of GalNAc, which were possibly resulted from exonuclease and *β-N*-acetylglucosaminidase activities, respectively.