A Non-targeted 4D-Metabolomics Workflow for Quality Control of Chiral Heparin Disaccharide by LC-timsTOF and MetaboScape®

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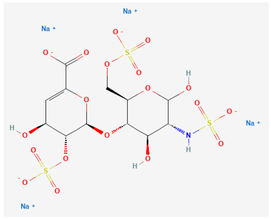
Introduction

Heparin is a highly sulfonated polysaccharide applied in the clinic for treatment of several diseases. Heparin disaccharide I-S tetrasodium salt purchased from various vendors have shown differences in target-binding activity as large as 30-fold, although 100% HPLC purity is provided by vendor's certificate of analysis (COA). The biological activity is thought to be closely related to its molecular structure, e.g., chirality. Trapped ion mobility spectrometry (TIMS) has the ion mobility separation power to differentiate chirality of heparin disaccharide. MetaboScape® is an integrated software supporting non-targeted screening, compound identification, and statistics. In this study, a 4D-Metabolomics data acquisition and analysis workflow is used to determine the differences among heparin disaccharide samples with ranging target-binding activities, which can provide a guidance for quality control of the production of this material.

Methods

Heparin disaccharide I-S tetrasodium salt standards were purchased from five different vendors. 1.0 mg/mL stock solutions of each sample were prepared in water/acetonitrile (50:50). Sample solutions were used for both direct infusion (acidified with 0.1% formic acid) and reverse phase liquid chromatography. LC-MS acquisitions were performed by Elute UHPLC with a C18 column and timsTOF Pro 2 (Bruker) with TIMS enabled in positive negative modes. Data analysis was conducted in DataAnalysis 5.3 (Bruker). Non-targeted feature detection was achieved by MetaboScape® 2022b (Bruker). Principal component analysis (PCA) and partial least squares regression (PLS) were performed to identify possible contaminant compounds which are trending with reduced biological activity. Compounds were annotated with SmartFormula and spectral libraries, e.g., MetaboBase 3.0. Additionally, data was assessed for isomeric components by acquired mobilograms and CCS values.



Samples	Relative Activity
Sigma	70
A	10
B	5
C	3
D	2

Fig. 1 Molecular structure of heparin disaccharide I-S tetrasodium salt. Samples purchased from different vendors provided various target-binding activities.

Accurate Mass by QToF

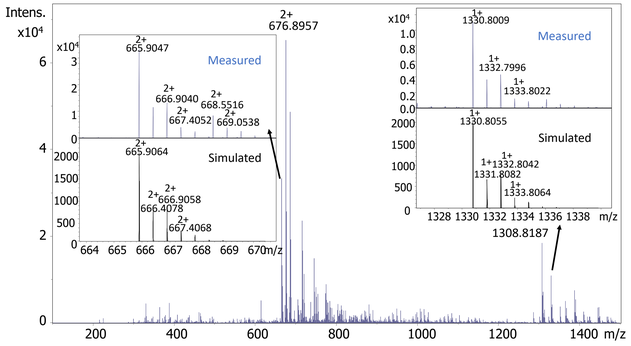


Fig. 2 Mass spectrum of heparin disaccharide by direct injection. Measured and simulated isotopic distribution for singly (1330.8 *m/z*) and doubly charged (665.9 *m/z*) ions of the dimer of heparin disaccharide I-S sodium salt is shown in the insets. Mass accuracy of <5 ppm was achieved.

Feature Annotation by MetaboScape®

	RT [min]	m/z meas.	M meas.	Ions	MS/MS	Name	Molecular Formula	Δm/z [ppm]	mSigma	Mob. 1/K0	CCS (Å²)	Annotations	AQ	Annotation Source
1	5.16	235.16927	234.16199	[M+H]+	As	3,5-Di-tert-butyl-2-hydroxybenzaldehyde	$C_{15}H_{22}O_2$	0.428	8.0	0.766	162.3			Bruker NIST 2020 MSMS Spectral Library_hr
2	2.87	283.17505	282.16778	[M+H]+	As	Hexaethylene glycol	$C_{12}H_{26}O_7$	-0.274	1.9	0.792	166.3			Bruker NIST 2020 MSMS Spectral Library_hr
3	0.69	300.03964	301.04692	[M+H]+	As	N-Acetyl-D-galactosamine 4-sulfate	$C_8H_{15}NO_9S$	0.306	10.8	0.565	118.4			Bruker NIST 2020 MSMS Spectral Library_hr
4	0.69	258.02886	259.03614	[M+H]+	As	Glucosamine 6-sulfate	$C_6H_{13}NO_8S$	-0.054	10.4	0.693	146.1			Bruker HMDB Metabolite Library_2.0
5	0.65	641.90951	642.91679	[M+H]+	As		$C_{12}H_{19}NNaO_{17}S_3$	0.029	18.7	1.023	209.3			
6	0.67	619.92744	620.93471	[M+H]+	As		$C_{12}H_{17}NNa_2O_{17}S_3$	-0.084	15.3	0.645	132.0			
7	0.68	458.95200	459.95928	[M+H]+	As		$C_{12}H_{13}N_2NaO_{14}PS_2$	-1.002	3.7	0.600	123.8			
8	0.66	309.46063	620.93582	[M+2H]2+	As		$C_{12}H_{17}NNa_2O_{17}S_3$	1.368	8.3	0.612	250.5			
9	0.67	298.46952	598.95359	[M+2H]2+	As		$C_{12}H_{22}NNa_2O_{14}PS_3$	1.290	9.9	0.647	265.1			
10	0.68	298.46981	598.95418	[M+2H]2+	As		$C_{12}H_{21}NNa_3O_{14}PS_3$	-1.785	16.9	0.603	247.0			
11	0.70	597.94547	598.95275	[M+H]+	As		$C_{12}H_{22}NNa_2O_{14}PS_3$	0.049	6.8	0.997	204.3			
12	0.70	597.94547	598.95274	[M+H]+	As		$C_{12}H_{21}NNa_3O_{14}PS_3$	-0.557	2.9	0.772	158.2			
13	0.73	496.00698	515.02482	[M+NH4]+	As		$C_{12}H_{25}N_2NaO_{14}PS_3$	0.355	6.4	1.015	208.9			
14	0.73	575.96367	576.97095	[M+H]+	As		$C_{12}H_{23}NNa_2O_{14}PS_3$	0.289	5.9	1.014	208.0			

Fig. 3 Screen shot of a feature table with selected features annotated by spectral libraries and SmartFormula in MetaboScape®.

Ion Mobility by TIMS

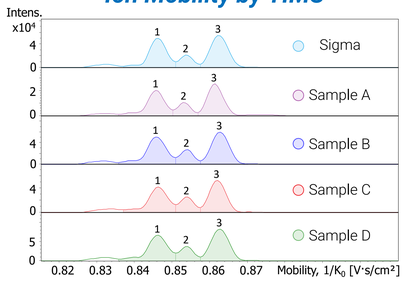


Fig. 4 Ion mobility separation of heparin disaccharide at 665.9 *m/z* by direct injection using optimized TIMS settings. Three chiral compounds are separated with resolution R > 0.8.

	Mobilogram Peak Area			
Samples	Chirality 1	Chirality 2	Chirality 3	Ratio
Sigma	282.6	93.4	311.3	1 : 0.3 : 1.1
A	117.4	45.4	129.8	1 : 0.4 : 1.1
B	327.3	118.6	336.7	1 : 0.4 : 1.0
C	264.0	115.1	279.6	1 : 0.4 : 1.1
D	436.2	170.6	478.4	1 : 0.4 : 1.1

Fig. 5 The ratio of 3 chiral heparin disaccharide compounds is similar among the samples with high and low biological activities.

Results

- Singly and doubly charged ions of the dimer of heparin disaccharide are measured within 5 ppm *m/z* error when the analyte solution was introduced into timsTOF Pro 2 by direct infusion using a syringe pump (Fig. 2)
- Three chiral isomers are observed from heparin disaccharide (Fig. 4).
- The ratio of 3 chiral isomers are similar regardless of different vendor and biological activities, indicating the chirality of heparin disaccharide is not responsible for activity (Fig. 5).
- Unsupervised statistical analysis, PCA, of heparin disaccharide shows a clear separation between samples from Sigma and other vendors. More than 50% variance can be explained by PC1 and PC2 (Fig. 6).
- Supervised statistical analysis, PLS, of heparin disaccharide helps identify features trending or unrelated with biological activities (Fig. 7).
- Features of interest can be filtered and annotated by spectral libraries and SmartFormula using MetaboScape® (Fig. 3).

Non-targeted Data Analysis by MetaboScape®

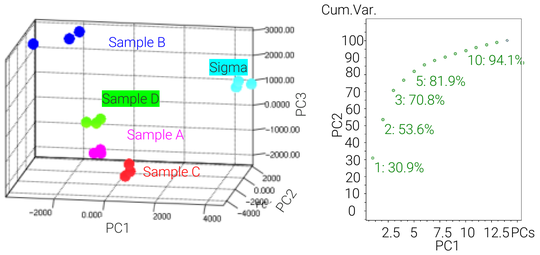


Fig. 6 Principal component analysis (PCA) 3-D scores (left panel) and explained variance plot heparin disaccharide samples using a non-targeted 4D-metabolomics workflow.

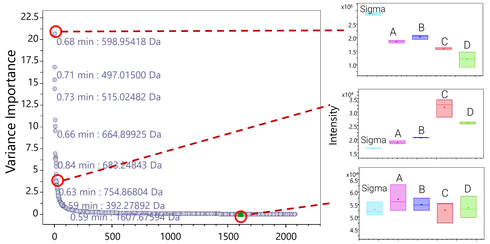


Fig. 7 Partial least squares regression (PLS) analysis variance importance plot (left panel) and intensity of 3 representative features (right panel) trending (top), reverse-trending (middle), and unrelated (bottom) with biological activity.

Conclusion

- TIMS can separate chiral compounds of heparin disaccharide, but chirality is not responsible for its biological activity
- Non-targeted 4D-metabolomics workflow can be used to identify features trending with biological activity using MetaboScape®
- Features annotated by MetaboScape® can further assist in quality control of manufacturing heparin disaccharide I-S tetrasodium salt

TIMS and Non-targeted 4-D Metabolomics