A Fast and Robust Quantitative Assay for Dekaparin in Dog Plasma by Heparan Lyase Digestion Coupled to UPLC-MS/MS

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Introduction

- Dekaparin is an active oligosaccharide with a molecular weight of 3520 Da, which is under investigation to be used as an anticoagulant drug to replace animal-sourced low-molecular weight heparin.
- Dekaparin is composed of heparan sulfate (HS) dodecasaccharide (12-mer) carrying 16 sulfate groups (**Figure 1**). HS is an oligosaccharide with a repeating unit of either iduronic acid (IdoA) or glucuronic acid (GlcA) and glucosamine (GlcN) residues; each anticoagulant drug residue is capable of carrying sulfate groups. These sulfation patterns govern its binding to growth factors, protein inhibitors, and chemokines, to regulate embryonic development and control blood coagulation and inflammatory responses.
- Due to the relatively large molecular weight of dekaparin, its multiple sulfate groups and high polarity from these sulfate groups, developing a reliable and selective quantitative assay in a biological matrix proved challenging. In this presentation, we report a fast, high-throughput, simple, and robust assay to quantify Dekaparin in dog plasma by heparan lyase digestion coupled to UPLC-MS/MS using ^{13}C-labeled Dekaparin as an internal standard (**Figure 1**).

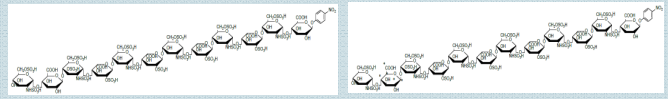

Figure 1: Chemical structures of dekaparin (*left*) and ^{13}C-dekaparin (*right*).

Method Challenges

Due to the high polarity and the relatively large molecular weight, it is very challenging to develop a robust LC-MS/MS method for the quantification of dekaparin. In this method, the intact dekaparin was qualitatively confirmed by Q-TOF mass spectrometry (**Figure 2**) based on the information of its sodium adducts and desulfonation (**Figure 3**).

Sample preparation was conducted by digesting dekaparin by heparan lyase to cleave oligomers into triglyceride residues prior to UPLC-MS/MS analysis (**Figure 4**). The digestion efficiency of heparan lyase was investigated for different parameters, such as the types of brand, concentrations of digestion solution, and times of digestion. An example of the digestion efficiency versus digestion times is shown in **Figure 5**. The extraction efficiency was evaluated using different extraction approaches, including protein precipitation extraction (PPE), liquid-liquid extraction, and solid-phase extraction; the PPE method was adopted in this assay.

Triple-quadrupole MS was then used for quantitation using an optimized ion source temperature (**Figure 6**). Cross-talking between dekaparin and its internal standard (IS) was encountered and resolved by adding dummy transitions (**Table 1**). Various HPLC columns were also screened, and the XBridge™ Amide column (Waters, Milford, MA) was selected. Different mobile phases were explored to achieve high quality chromatography, and the mixture of acetonitrile and water with additives of hexafluoroisopropanol (HFIP) and benzotriazole (BTA) showed symmetric and reproducible peaks with a sufficient resolution and low baseline. In order to minimize carryover, optimized column and needle wash solutions consisting of acetonitrile, methanol, isopropanol, formic acid, and water were used (**Figure 7**).

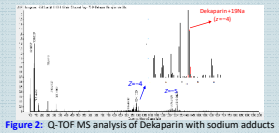

Figure 2: Q-TOF MS analysis of Dekaparin with sodium adducts

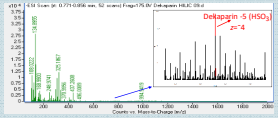

Figure 3: Q-TOF MS analysis of dekaparin with desulfonation

Figure 4: Heparan lyase digestion process in sample preparation

Compound	Parent Ion (*m/z*)	Product Ion (*m/z*)	Dwell Time (ms)
Dekaparin	595.1	416.0	200
Dummy-1	595.1	258.0	20
^{13}C-Dekaparin	601.1	258.0	200
Dummy-2	601.1	422.0	20

Table 1: TripleQuad Multiple Reaction Monitoring (MRM) MS parameters

Method Challenges (Cont.)

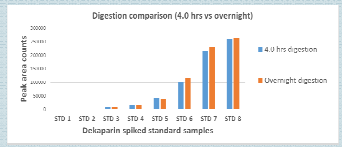

Figure 5: Comparison of heparan lyase digestion times

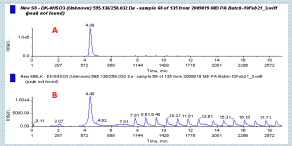

Figure 7: Chromatogram **A** shows optimized conditions without carryover; Chromatogram **B** shows original conditions with severe carryover

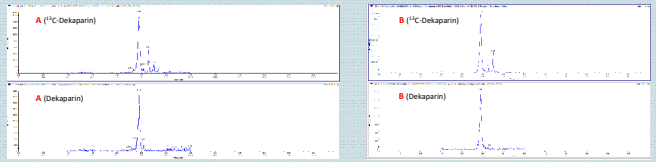

Figure 6: Comparison of sensitivity versus ion source temperature – Chromatogram **A**, 500 °C; Chromatogram **B**, 700 °C

Finalized Method

Dog plasma (K_2EDTA) samples containing Dekaparin and ^{13}C-dekaparin (internal standard [IS]) were initially digested for 4 hours at room temperature using Heparan Lyase Enzyme Cocktail, which is composed of heparan lyases I, II, and III, and then extracted using acetonitrile/methanol (50:50, *v/v*). The extracts were analyzed on a Waters XBridge Amide column (3.5 µm, 2.1 x 100 mm) at 30°C. The mixed mobile phase solutions including HFIP, BTA, water, and acetonitrile were used with a shallow gradient at 0.5 mL/min for 6.5 minutes.

A SCIEX API 6500 UPLC-MS/MS system was used in ESI (electrospray ionization) mode under negative ions to monitor *m/z* 595.1→416.0 and 601.1→258.0 transitions of dekaparin and IS, respectively. The diverter valve of the UPLC-MS/MS system was used ~~in~~ to reduce ~~the~~ contamination of the system.

Results

The UPLC-MS/MS assay was successfully developed and fully validated in dog plasma within a quantification range of 1.00 to 100 µg/mL (**Figure 8**). The validation experiments included intra-day and inter-day precision and accuracy, sensitivity, selectivity/specificity, matrix effect, various stability tests, recovery, and dilution linearity. Typical chromatograms of the lower limit of quantification quality control (LLOQ-QC), upper limit of quantification QC (ULOQ-QC), matrix blank, and control-zero are shown in **Figure 9**. The intra-day accuracy (% bias) results for three runs of LLOQ and other QCs were within 80.0–120% and 85.0–115%, respectively; the intra-day precision (%CV) results for three runs of LLOQ and other QCs were within 20.0% and within 15.0%, respectively (**Table 2**). Selectivity was successfully determined without observable interference using six different lots of matrix. Seventeen (17) hours of benchtop matrix stability, four (4) cycles of freeze-thaw stability, and 1 month (36 days) of long-term matrix stability at −70°C were successfully established. One hundred and sixty-five (165) hours of processed sample stability was established at 2–8°C (**Table 3**). Dilution linearity was successfully evaluated for 10 times dilution at a concentration of 400 µg/mL. This validated method was successfully applied to a preclinical pharmacokinetic study.

Results (Cont.)

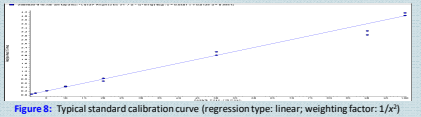

Figure 8: Typical standard calibration curve (regression type: linear; weighting factor: $1/x^2$)

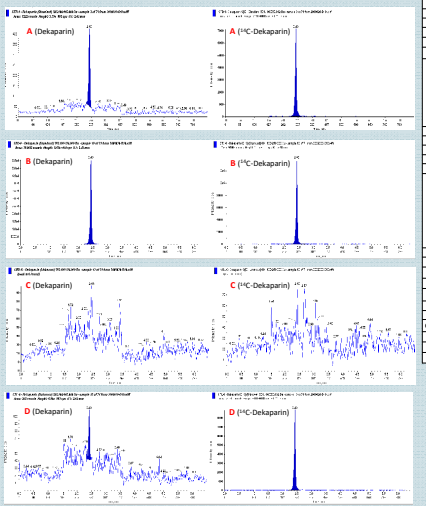

Figure 9: Typical chromatograms of LLOQ-QC (**A**), ULOQ-QC (**B**), matrix blank (**C**), and control-zero (**D**) samples.

Table 2: Inter- and intra- accuracy and precision

Batch ID	LLOQ-QC (ng/mL)	DEV	LQC (ng/mL)	DEV	MQC (ng/mL)	DEV	HQC (ng/mL)	DEV
01	1.00	(%)	3.00	(%)	15.0	(%)	80.0	(%)
	1.00	3.8	2.96	7.6	16.0	-1.8	82.2	-1.2
	0.760	-21.1	3.13	13.8	15.6	-4.3	78.8	-5.3
	0.963	0.0	2.75	0.0	16.3	0.0	83.2	0.0
	0.808	-16.1	2.77	0.7	16.6	1.8	85.3	2.5
	0.832	-13.6	2.75	0.0	16.0	-1.8	83.2	0.0
	0.841	-12.7	3.07	11.6	14.8	-9.2	79.7	-4.2
n	6		6		6		5	
Mean	0.867		2.91		15.9		82.1	
SD	0.0935		0.172		0.627		2.42	
CV(%)	10.8		5.9		3.9		2.9	
Accuracy(%)	86.7		97.0		106.0		102.6	
02	0.826	-14.2	3.04	10.5	14.7	-9.8	77.6	-6.7
	0.829	-13.9	2.99	8.7	15.2	-6.7	79.4	-4.6
	0.865	-10.2	2.73	-0.7	15.6	-4.3	80.9	-2.8
	0.969	0.6	3.46	25.8	14.2	-12.9	78.7	-5.4
	0.686	-28.8	2.62	-4.7	15.0	-8.0	76.6	-7.9
	0.883	-8.3	2.57	-6.5	14.8	-9.2	78.2	-6.0
n	6		6		6		6	
Mean	0.843		2.90		14.9		78.6	
SD	0.0928		0.334		0.475		1.49	
CV(%)	11.0		11.5		3.2		1.9	
Accuracy(%)	84.3		96.7		99.3		98.3	
3	0.735	-23.7	2.57	-6.5	11.7	-28.2	59.9	-28.0
	0.824	-14.4	2.25	-18.2	11.9	-27.0	59.6	-28.4
	0.933	-3.1	2.22	-19.3	13.2	-19.0	66.2	-20.4
	0.791	-17.9	2.48	-9.8	11.5	-29.4	66.0	-20.7
	0.917	-4.8	2.57	-6.5	16.7	2.5	76.1	-8.5
	0.877	-8.9	2.35	-14.5	13.3	-18.4	72.0	-13.5
n	6		6		6		6	
Mean	0.846		2.41		13.1		66.6	
SD	0.0767		0.156		1.95		6.54	
CV(%)	9.1		6.5		14.9		9.8	
Accuracy(%)	84.6		80.3		87.3		83.3	
Inter-assay:								
n	18		18		18		18	
Overall mean	0.852		2.738		14.617		75.756	
SD	0.083		0.327		1.662		7.821	
CV(%)	9.7		11.9		11.4		10.3	
Accuracy(%)	85.2		91.3		97.4		94.7	

Table 3: Stability data

Parameter	Established Stability
Long-term storage at – 20°C	36 days
Benchtop room temperature (RT) storage	17 hours
Processed sample stability at 4°C	165 hours
Freeze/thaw (– 20°C/RT) stability	4 cycles
Working solution stability at – 20°C	63 days

Conclusions

A fast, simple, and robust assay using heparan lyase digestion coupled to UPLC-MS/MS assay was developed and validated to quantify the concentration of dekaparin in dog plasma (K_2EDTA) within an assay range of 1.00 to 100 µg/mL. The assay has the following advantages:

- ***Simplicity:*** The heparan lyase digestion was adopted to cleave the dekaparin to a unique triglyceride residue used for quantification in this assay. In comparison to a traditional overnight digestion step, the 4-hour process of heparan lyase digestion was optimized. Subsequently, a simple protein precipitation extraction was applied to purify the sample.
- ***High-Quality Chromatography:*** The multiple sulfate groups in Dekaparin result in tailing peaks on the UPLC-MS/MS chromatogram. A Waters™ XBridge Amide column and a mobile phase composed of acetonitrile, water, HFIP, and BTA were used in this assay to provide symmetric peaks with a sufficient resolution and low baseline.
- ***Precision and Accuracy:*** This assay was fully validated in accordance with U.S. Food and Drug Association regulations and showed good precision and accuracy.
- ***Higher Throughput:*** The total elapsed time to process one full 96-well plate was ~6 hours. The total chromatography run time was 6.5 minutes per sample.