Quantification of the Polydisperse Heparin Analog Dociparstat by Signature Disaccharide Analysis in the Presence of Concomitant Enoxaparin Dosing in Support of a Phase 2/3 Study in Patients with Severe COVID-19

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OVERVIEW

Analysis of a polydisperse heparin analog therapeutic (CX-01, Dociparstat) was conducted in human plasma by signature disaccharide (NS6S) analysis, with NS6S being unique to CX-01 in native human plasma but common to the concomitantly dosed low molecular weight heparin (LMWH), Enoxaparin, in COVID-19 patients, demonstrating a novel quantification approach to concomitant related glycosaminoglycan (GAG) therapeutics. Signature disaccharide response correction was required to achieve accurate Dociparstat quantification in concomitantly dosed samples by in-run monitoring of an alternate signature disaccharide unique to the LMWH. The method was used to determine CX-01 concentrations in a Phase 2/3 study to evaluate the safety and efficacy of Dociparstat sodium for the treatment of severe COVID-19 in adults at high risk of respiratory failure.

INTRODUCTION

Bioanalytical methodologies were previously developed, extensively optimized, and validated at Q² Solutions for quantification of the novel CX-01 therapeutic GAG in rat, dog and human plasmas. The core methodology was based on concepts from the literature works of Linhardt[1], et al., focusing on the analysis of disaccharides derived from GAG macromolecule hydrolysis.

Key Optimization Aspects for Successful CX-01 Method Validations:

- GAG hydrolysis, signature disaccharide selectivity confirmation, disaccharide derivatization, HRMS detection with stable label ISTD disaccharide ($NS6S\text{-}^{13}C_6$)

Key Development Aspects for CX-01 Analysis in the Presence of Enoxaparin:

- Response correction in the previously validated CX-01 method for “added” NS6S detected from patients receiving concomitant CX-01 and Enoxaparin treatments, where both treatment materials contain NS6S disaccharide subunits
    - Identification of a unique Enoxaparin disaccharide (NS2S) hydrolysis product
    - Derivation of NS6S-to-NS2S response relationship over the relevant Enoxaparin range
    - Compensation for non-equivalent Enoxaparin hydrolysis and derivatization efficiencies under CX-01 optimized conditions
        - Run specific curve fitting

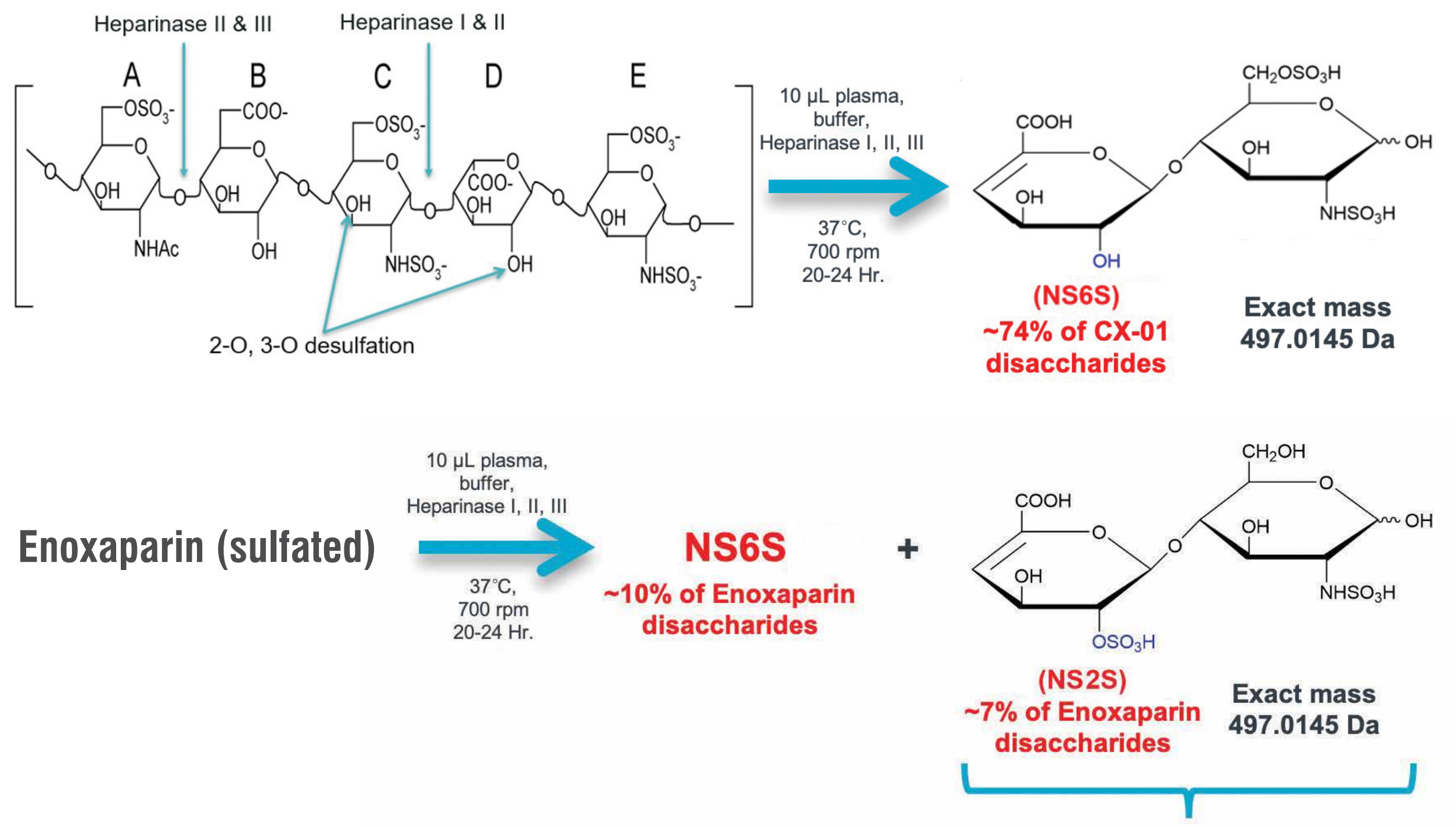
METHOD

The method described quantifies CX-01 in K_2EDTA human plasma over a target assay range of 0.0500 – 50.0 µg/mL in the presence of concomitantly dosed Enoxaparin. The effective CX-01 concentration range is determined on a run-by-run basis and by the measured quality of signature disaccharide (NS6S) response correction near the low end of the assay by including in-run concomitantly spiked QC samples. The method is based on the enzymatic hydrolysis of CX-01 and Enoxaparin in human plasma to generate signature disaccharides for the primary and concomitant analytes of interest, the addition of stable label ISTD disaccharides, protein precipitation of the hydrolysis mixture, derivatization[2,3] of signature disaccharides and ISTDs, and LC-HRMS in PRM mode for instrumental analysis. Subsequent response correction for the concomitantly dosed Enoxaparin is conducted as described herein to arrive at an accurate target therapeutic (CX-01) concentration determination.

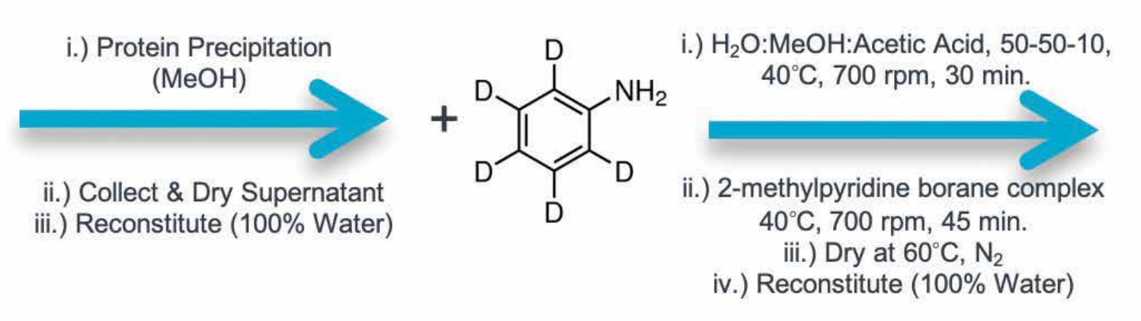
METHODOLOGY OVERVIEW:

i.) Sample Hydrolysis

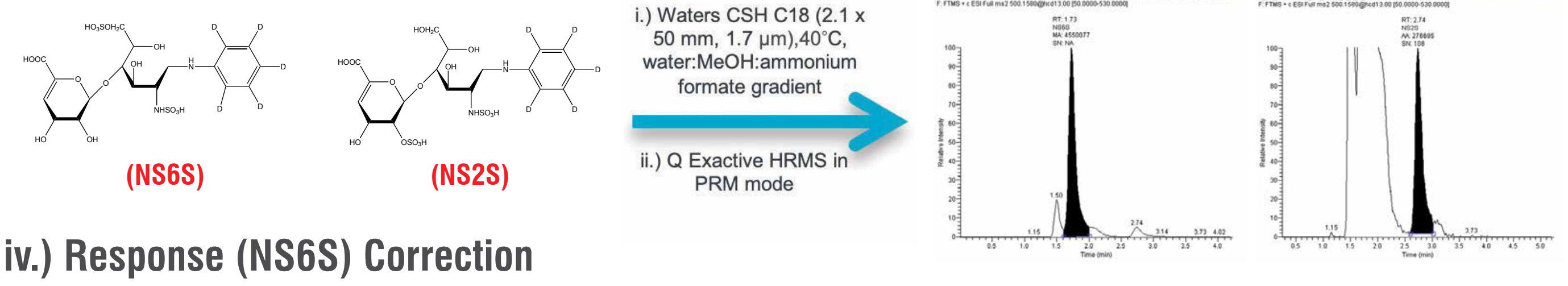
CX-01 Structure



ii.) Add $NS6S\text{-}^{13}C_6$ + $NS2S\text{-}^{13}C_6$



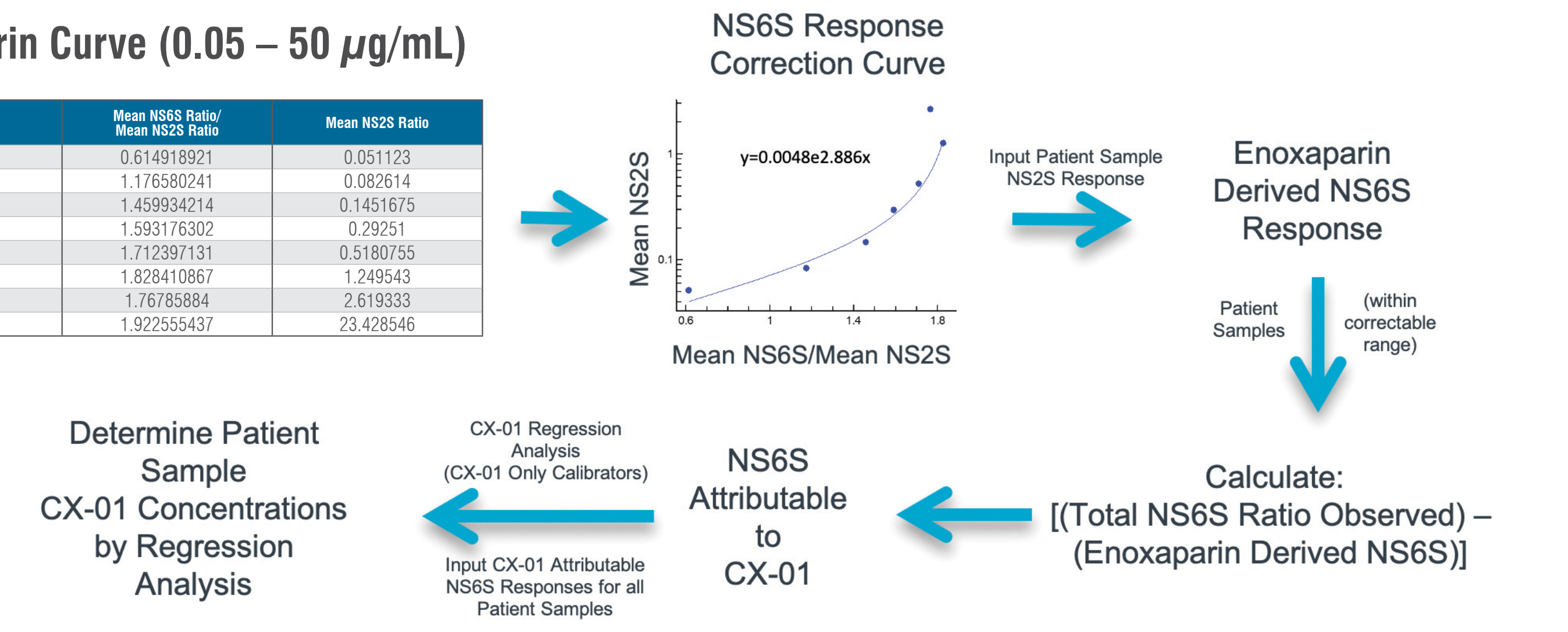
iii.) Separation and Detection



iv.) Response (NS6S) Correction

Enoxaparin Curve (0.05 – 50 µg/mL)

Enox. (µg/mL)	Mean NS6S Ratio/ Mean NS2S Ratio	Mean NS2S Ratio
0.05	0.614918921	0.051123
0.1	1.176580241	0.082614
0.25	1.459934214	0.1451675
0.5	1.593176302	0.29251
1	1.712397131	0.5180755
2.5	1.828410867	1.249543
5	1.76785884	2.619333
50	1.922555437	23.428546



RESULTS

The method was qualified in a single run. Assay performance demonstrated individual calibration standard %biases ranging from -6.0% - +6.0% for the 8-point CX-01 calibration line assayed in duplicate (Table 1). A mean %bias of -2.0% to +0.5% was observed across four levels of CX-01 QCs (Table 2). Three additional QCs, designed to characterize individual NS6S response correction, were prepared at low (0.150 µg/mL) and high (40.0 µg/mL) CX-01 concentrations to also contain 0.500 µg/mL, 2.00 µg/mL, or 10.0 µg/mL Enoxaparin (Table 3). These additional QCs demonstrated NS6S response corrected %biases from -1.3% to -13.3% for samples containing up to 2.00 µg/mL Enoxaparin. The low QC %bias exceeded correctability tolerances at the 10.0 µg/mL Enoxaparin level. For the high QC samples, %bias ranged from +3.5% to +5.5% across the concentrations of Enoxaparin tested.

Additionally, an eight-point CX-01 calibration line spanning the target assay range (0.0500 – 50.0 µg/mL) and containing an ~Cmax Enoxaparin level (5 µg/mL), was assayed and subjected to in-run NS6S response correction. Corrected NS6S response values for individual combinations spiked calibration standards demonstrated 87% – 103% of the observed response from CX-01 - only spiked calibrators prepared at the same nominal levels over a CX-01 range of 0.25 -50 µg/mL (Table 4). Corrected response values across the two lowest CX-01 calibrator levels (0.0500 – 0.100 µg/mL) ranged from -137% to +169%, demonstrating an expected limitation to the response correction applied.

Additional method qualification parameters assessed included room temperature Enoxaparin benchtop stability in plasma and potential for variability in assay performance when processing Enoxaparin from various sources/vendors. The ~25-hour benchtop stability samples demonstrated responses ranging from 89.4% to 103.2% of fresh preparations (Table 5). Observed NS6S ratios from Enoxaparin supplied by four different vendors varied from +105% to +153% of the mean observed ratios from a US Pharmacopeia standard (Table 6).

Three runs were conducted for non-regulated sample analysis of 66 phases 2/3 clinical samples in patients with severe COVID-19 using the qualified method that met the acceptance criteria for passing runs. Incurred sample reproducibility (ISR) was also assessed by the assay of at least 10% of the study samples. All paired original and repeat assay results for the ten samples undergoing ISR analysis were within the acceptable limits (+/- 20.0%) for CX-01.

Method Qualification and Phase 2/3 Study ISR Results:

Table 1. Method Qualification Run - CX-01 Calibration line Results

Qual. Curve Number	CX-01 0.0500 µg/mL	%Bias	CX-01 0.100 µg/mL	%Bias	CX-01 0.250 µg/mL	%Bias	CX-01 0.500 µg/mL	%Bias	CX-01 1.00 µg/mL	%Bias	CX-01 2.50 µg/mL	%Bias	CX-01 5.00 µg/mL	%Bias	CX-01 50.0 µg/mL	%Bias
5	0.0506	1.2	0.101	1	0.258	3.2	0.499	-0.2	1.02	2	2.57	2.8	5.3	6	49.5	-1
	0.0505	1	0.094	-6	0.254	1.6	0.482	-3.6	0.972	-2.8	2.36	-5.6	5.03	0.6	50.2	0.4

Table 2. Method Qualification Run - CX-01 QC/VS Results

Curve Number	LOW VS 0.100 µg/mL	%Bias	LOW-MIDDLE VS 1.00 µg/mL	%Bias	MIDDLE VS 5.00 µg/mL	%Bias	HIGH VS 40.0 µg/mL	%Bias
5	0.0972	-2.8	0.949	-5.1	4.84	-3.2	39.5	-1.3
	0.0988	-1.2	0.992	-0.8	5.08	1.6	40.8	2
Intrarun Mean	0.098		0.971		4.96		40.2	
Intrarun %Bias	-2		-2.9		-0.8		0.5	

Table 3. Method Qualification Run - CX-01 + Enoxaparin Response Correction QC/VS Results

Curve Number	LOW VS 0.150 µg/mL CX-01	%Bias	LOW VS 0.150 µg/mL CX-01	%Bias	LOW VS 0.150 µg/mL CX-01	%Bias	HIGH VS 40.0 µg/mL CX-01	%Bias	HIGH VS 40.0 µg/mL CX-01	%Bias	HIGH VS 40.0 µg/mL CX-01	%Bias
	0.500 µg/mL Enoxaparin		2.00 µg/mL Enoxaparin		10.0 µg/mL Enoxaparin		0.500 µg/mL Enoxaparin		2.00 µg/mL Enoxaparin		10.0 µg/mL Enoxaparin	
5	0.149	-0.7	0.118	-21.3	0.136	-9.3	41	2.5	42.5	6.3	41.7	4.3
	0.146	-2.7	0.141	-6	-0.15	-200	41.7	4.3	41.9	4.8	42.6	6.5
Intrarun Mean	0.148		0.13		-0.007		41.4		42.2		42.2	
Intrarun %Bias	-1.3		-13.3		-104.7		3.5		5.5		5.5	

RESULTS (CONTINUED)

Table 4. Method Qualification Run - CX-01 + ~Cmax Enoxaparin – Correction Results

Calibration Standard (CS) CX-01 (Enoxaparin) µg/mL	Sample Name	Total NS6S Peak Area Ratio Detected (CX-01 + Enoxaparin)	Enoxaparin Attributable NS6S Peak Area Ratio (via NS2S Detection)	CX-01 Attributable NS6S Corrected Peak Area Ratio	CX-01 Only Spiked CS NS6S Peak Area Ratio	NS6S Correction Performance (Corrected NS6S Ratio from Co-dosed CS as a % of Observed NS6S Ratio from CX-01 only CS)
0.0500 (5.00)	E5 CX01 0.05 1	4.840544	4.5864078	0.2541362	0.220025	116%
0.100 (5.00)	E5 CX01 0.1 1	5.103162	4.333787628	0.769374372	0.455863	169%
0.250 (5.00)	E5 CX01 0.25 1	5.646511	4.468077229	1.178433771	1.192193	99%
0.500 (5.00)	E5 CX01 0.5 1	6.755343	4.726180079	2.029162921	2.321934	87%
1.00 (5.00)	E5 CX01 1 1	9.233416	4.592040994	4.641375006	4.758301	98%
2.50 (5.00)	E5 CX01 2.5 1	15.425792	4.720178338	10.70561366	12.008807	89%
5.00 (5.00)	E5 CX01 5 1	27.098882	4.638346279	22.46053572	24.835572	90%
50.0 (5.00)	E5 CX01 50 1	241.41663	4.465841734	236.9507883	233.029763	102%
50.0 (5.00)	E5 CX01 50 2	244.670275	4.390883916	240.2793911	236.251495	102%
5.00 (5.00)	E5 CX01 5 2	27.501933	4.767504469	22.73442853	23.563356	96%
2.50 (5.00)	E5 CX01 2.5 2	16.099827	4.835878542	11.26394846	11.049215	102%
1.00 (5.00)	E5 CX01 1 2	8.776138	4.555288266	4.220849734	4.540538	93%
0.500 (5.00)	E5 CX01 0.5 2	6.985195	4.715777189	2.269417811	2.240951	101%
0.250 (5.00)	E5 CX01 0.25 2	5.656462	4.45157086	1.20489114	1.172627	103%
0.100 (5.00)	E5 CX01 0.1 2	4.917385	4.883703902	0.033681098	0.423688	8%
0.0500 (5.00)	E5 CX01 0.05 2	4.637338	4.937657828	-0.300319828	0.219573	-137%

Table 5. Method Qualification Run - Enoxaparin Benchtop Stability Results

Sample Name	Enoxaparin (µg/mL)	NS6S Peak Area Ratio	Mean NS6S Peak Area Ratio (per supplier and level)	NS6S Ratio as % of Mean USP Material
BT 24Hr Enox 0.1 1	0.100	0.091968	-	96.2%
BT 24Hr Enox 0.1 2	0.100	0.091258	-	89.4%
BT 24Hr Enox 1 1	1.00	0.915588	-	103.2%
BT 24Hr Enox 1 2	1.00	0.911131	-	102.7%
BT 24Hr Enox 5 1	5.00	4.453483	-	94.6%
BT 24Hr Enox 5 2	5.00	4.1406	-	93.9%
USP Enoxaparin 0.1 1	0.100	0.099826	0.097202	
USP Enoxaparin 1 1	1.00	0.887151	0.887151	
USP Enoxaparin 5 1	5.00	4.750316	4.630611	
USP Enoxaparin 0.1 2	0.100	0.094578	-	
USP Enoxaparin 1 2	1.00	5.289682*	-	
USP Enoxaparin 5 2	5.00	4.510906	-	
* Note: Outlier value not included in mean response calculation.				

Table 6. Method Qualification Run - Enoxaparin Source/Vendor Results

Sample Name	Enoxaparin (µg/mL)	NS6S Peak Area Ratio	Mean NS6S Peak Area Ratio (per supplier and level)	NS6S Ratio as % of USP Standard
Amphastar ONLY 0.1 1	0.100	0.131748	0.1321675	136%
Amphastar ONLY 1 1	1.00	1.102758	1.1424365	129%
Amphastar ONLY 5 1	5.00	5.817375	5.7020395	123%
Amphastar ONLY 5 2	5.00	5.586704	-	
Amphastar ONLY 1 2	1.00	1.182115	-	
Amphastar ONLY 0.1 2	0.100	0.132587	-	
Winthrop ONLY 0.1 1	0.100	0.13243	0.1377545	142%
Winthrop ONLY 1 1	1.00	1.318801	1.355812	153%
Winthrop ONLY 5 1	5.00	5.30145	5.377683	116%
Winthrop ONLY 5 2	5.00	5.453916	-	
Winthrop ONLY 1 2	1.00	1.392823	-	
Winthrop ONLY 0.1 2	0.100	0.143079	-	
Lovenox ONLY 0.1 1	0.100	0.101129	0.104356	107%
Lovenox ONLY 1 1	1.00	0.951813	0.97016	109%
Lovenox ONLY 5 1	5.00	4.850046	4.845246	105%
Lovenox ONLY 5 2	5.00	4.840446	-	
Lovenox ONLY 1 2	1.00	0.988507	-	
Lovenox ONLY 0.1 2	0.100	0.107583	-	
USP Enoxaparin 0.1 1	0.100	0.099826	0.097202	-
USP Enoxaparin 1 1	1.00	0.887151	0.887151	-
USP Enoxaparin 5 1	5.00	4.750316	4.630611	-
USP Enoxaparin 0.1 2	0.100	0.094578	-	-
USP Enoxaparin 1 2	1.00	5.289682*	-	-
USP Enoxaparin 5 2	5.00	4.510906	-	-
* Note: Outlier value not included in mean response calculation.				

Table 7. Phase 2/3 Study ISR Results

Watson ID	Custom ID	Sample ID	Group	Subject	Treatment	Time Text	Final Analysis Run ID	Final Original Concentration (µg/mL)	ISR Run ID	ISR Concentration (µg/mL)	% Difference	Flag
11	6511803544-02	6511803544-02 0001-002 A Plasma-1 Day 4 / Day 4	1	0001-002	A	Day 4	3	3.03	6	2.65	-13.4	
34	6515496004-06	6515496004-06 0001-005 A Plasma-1 Early Therapy Discontinuation / Day 998	1	0001-005	A	Early Therapy Discontinuation	3	3.15	6	2.96	-6.22	
48	6511803599-06	6511803599-06 0001-007 A Plasma-1 Early Therapy Discontinuation / Day 998	1	0001-007	A	Early Therapy Discontinuation	3	5.82	6	5.92	1.7	
74	6511803593-02	6511803593-02 0002-002 A Plasma-1 Day 4 / Day 4	1	0002-002	A	Day 4	3	2.08	6	1.91	-8.52	
95	6511803591-02	6511803591-02 0002-005 A Plasma-1 Day 4 / Day 4	1	0002-005	A	Day 4	3	3.06	6	3.16	3.22	
96	6511803596-02	6511803596-02 0002-005 A Plasma-1 Day 8 / Day 8	1	0002-005	A	Day 8	3	3.47	6	3.51	1.15	
108	6511803636-02	6511803636-02 0003-003 A Plasma-1 Day 2 / Day 2	1	0003-003	A	Day 2	3	1.64	6	1.59	-3.1	
115	6512027790-02	6512027790-02 0007-001 A Plasma-1 Day 2 / Day 2	1	0007-001	A	Day 2	4	2.35	6	2.56	8.55	
116	6512027795-02	6512027795-02 0007-001 A Plasma-1 Day 4 / Day 4	1	0007-001	A	Day 4	4	2.12	6	2.15	1.41	
157	6511803638-02	6511803638-02 0015-001 A Plasma-1 Day 2 / Day 2	1	0015-001	A	Day 2	4	4.75	6	4.35	-8.79	
Overall: 100% of all repeats were within specification (Pass:10 Fail:0)												

CONCLUSIONS

CX-01 quantitation in the presence of concomitantly dosed Enoxaparin requires an in-run signature disaccharide response correction approach. The assay requires the determination of concomitant medication concentration via alternate disaccharide monitoring and subsequent correction of the total signature disaccharide response detected to arrive at an accurate target therapeutic concentration determination. Run-specific curve fitting, relative to co-dosed QC samples intended to mimic patient samples containing up to Cmax levels of Enoxaparin, as well as ISR results from a Phase 2/3 study for which the method was deployed, demonstrated reasonable response correction and highly reproducible CX-01 concentration determinations from concomitantly-dosed patient samples.

REFERENCES

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[3] S. Bawazeer, A.M. Ali, A. Alhawiti, A. Khalaf, C. Gibson, J. Tusiimire, D.G. Watson, Talanta, vol. 166, 1 May 2017, 75-80.