

Heparin Impurities Testing

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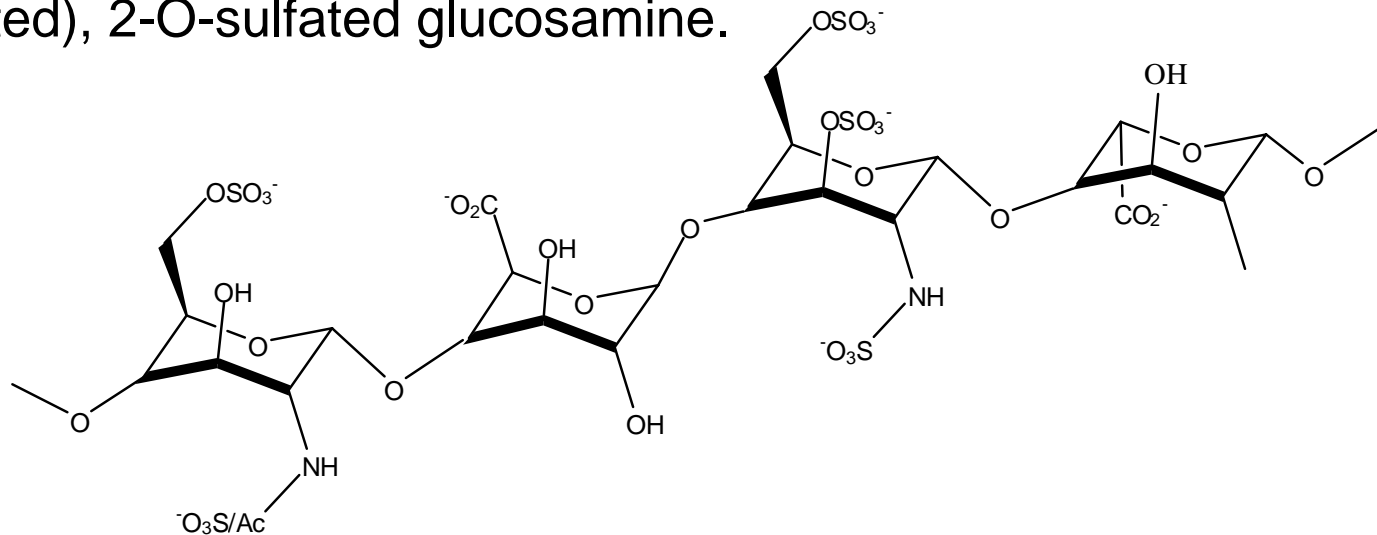
Baxter Healthcare Corporation

Starting Point

- Heparin is biologically derived molecule – variability in the starting material and finished API due to biological source.
- Heparin purification process employs enzyme (protease) digest, extremes of pH, oxidation/bleach steps, solvent precipitation.
- No regulatory guidance exists for heparins. ICH Q6B, *“Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products”*, excludes heparins.
- A heparin mimetic, over-sulfated chondroitin sulfates (OSCS), has been used to adulterate product.

Heparin molecule

- Repeating disaccharide units contain 2-O-sulfated iduronic acid and 6-N-sulfated, 2-O-sulfated glucosamine, along with disaccharides of glucuronic acid and 6-N-sulfated (or N-acetylated), 2-O-sulfated glucosamine.



- Negatively charged molecule
- Molecular weight range is 5,000 to 25,000 Da.
- Biosynthetic precursor is a proteoglycan with linkage from the carbohydrate via $\beta 1,4\text{-D-glucuronic acid} \rightarrow \beta 1,3\text{-D-galactose} \rightarrow \beta 1,3\text{-D-galactose} \rightarrow \beta 1,4\text{-D-xylose} \rightarrow \text{O-serine}$.

Source	Potential Impurity
Biological molecule	<ul style="list-style-type: none">• Residual peptide-carbohydrate linker (removed during purification process)• Dermatan sulfate• Chondroitin sulfate
Purification process	<ul style="list-style-type: none">• Oxidation, peroxidation by-products• Residual solvents• Residual proteins and peptides• Residual nucleic acids
Adulteration	<ul style="list-style-type: none">• OSCS

USP Heparin Monograph Changes Phase 1 to Phase 2



	USP Phase 1 (June 18, 2008)	USP Phase 2 (proposed)
Identification A	¹ H NMR Spectrum (@200:1 near 2 ppm, no detectable peaks between 2.12 – 3.00 ppm)	¹ H NMR Spectrum (@ 1000:1 near 2 ppm: <ul style="list-style-type: none"> •peaks at 5.42, 5.23, 3.28 (doublet), 2.05; •no unidentified peaks >4% of mean of 5.42 ppm and 5.23 ppm between 0.10-2.00, 2.10-3.00, 5.70-8.00; •no signal >200% of mean of 5.42 ppm and 5.23 ppm in 3.35-4.55 ppm
Identification B	Capillary electrophoresis Test matches standard with no sharp peak preceding heparin peak	Chromatographic identity Sample peaks match standard peaks
Identification C	Sheep plasma assay for heparin	Anti-factor Xa to anti-factor IIa ratio NLT 0.9 and NMT 1.1
Identification D	Flame test for Sodium	Flame test for Sodium
Assay	Sheep plasma NLT 140 U/mg 90 – 110 % label claim	Anti-factor IIa NLT 180 U/mg
	Anti-factor Xa activity (100 x anti-factor Xa / anti-coagulant potency) 80 – 120%	

USP Heparin Monograph Changes Phase 1 to Phase 2



	USP Phase 1 (June 18, 2008)	USP Phase 2 (proposed)
Inorganic impurities	Residue on ignition 28.0 – 41.0%	Residue on ignition 28.0 – 41.0%
	Heavy metals 0.003%	Heavy metals NMT 30 ppm
	Nitrogen content 1.3 – 2.5%	Nitrogen content 1.3 – 2.5%
Organic Impurities		Limit of galactosamine in total hexosamine NMT 1%
		Nucleotidic impurities @260 nm NMT 0.20
		Absence of OSCS (identification A and B)
	Protein No precipitate or turbidity in TCA	Protein NMT 1.0%
		Residual solvents meets <467>
Specific Tests	pH 5.0-7.5	pH 5.0-7.5
	Bacterial endotoxin NMT 0.03 USP EU / USP heparin U	Bacterial endotoxin NMT 0.03 USP EU / USP heparin U
	Sterility <71> Meets requirement	Sterility <71> Meets requirement
	Loss on drying NMT 5.0% weight loss	Loss on drying NMT 5.0% weight loss

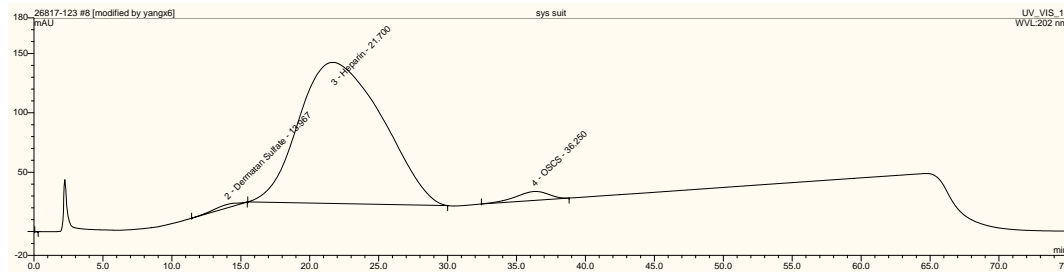
Brief description of the proposed method

- Instrument used: Dionex ICS 3000 with Chromeleon software
- Columns: Dionex IonPac AG11, 2 X 50 mm guard and AS11, 2 X 250 mm, analytical
- Mobile phase A: 0.8 g of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ /2 L, adjusted to pH 3.0 with H_3PO_4 .
- Mobile phase B: 0.8 g of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and 280 g of $\text{NaClO}_4 \cdot \text{H}_2\text{O}$ in 2 L, adjusted to pH 3.0 with H_3PO_4 .
- Flow rate: 0.22 mL/min, Injection volume: 10 uL
- UV at 202 nm
- Runtime: 75 min
- Retention times reported in PF: About 22 min (dermatan sulfate, DS), 30 min (heparin), and 50 min (OSCS)
- Resolution: NLT 1.0 between DS and Heparin, NLT 1.5 between Heparin and OSCS
- Acceptance criterion: Matching retention time of major peak (heparin)

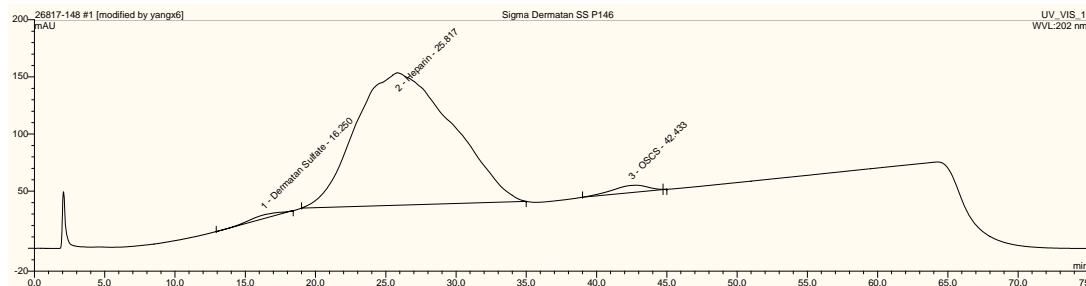
Method updates:

- Top: NaH_2PO_4 and NaClO_4 used for mobile phase preparation (PF 35(2))
- Bottom: $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and $\text{NaClO}_4 \cdot \text{H}_2\text{O}$ used for mobile phase preparation (per communication with USP)

Example Chromatograms of System Suitability Solution at Above Conditions



R (half height width)
DS/Hep: 1.0-1.1
Hep/OSCS: 1.8



R (half height width)
DS/Hep: 0.9-1.0
Hep/OSCS: 1.8-1.9

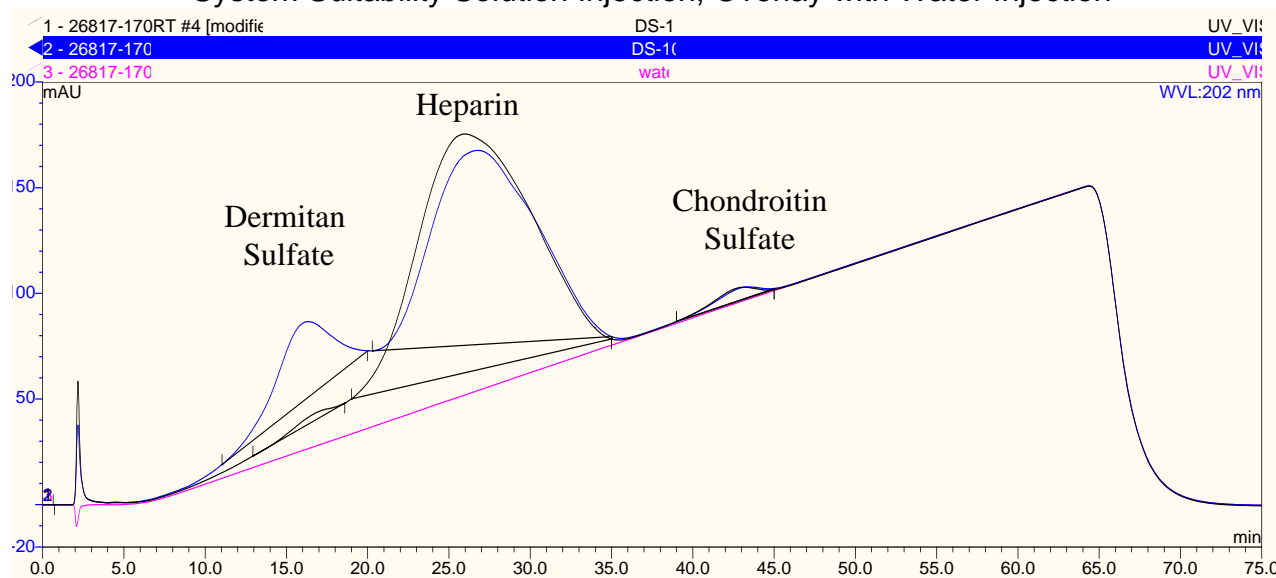
Heparin Chromatographic Identification Method

USP Resolution $R = \frac{2(t_2 - t_1)}{W_1 + W_2}$ or $R = \frac{2(t_2 - t_1)}{1.70 (W_{1,w/2} + W_{2,w/2})}$

(This equation is same as EP resolution and is a default equation in Dionex's Chromeleon software)

For the asymmetrical peaks, it is more appropriate to use equation based on peak width at half height.

System Suitability Solution Injection, Overlay with Water Injection



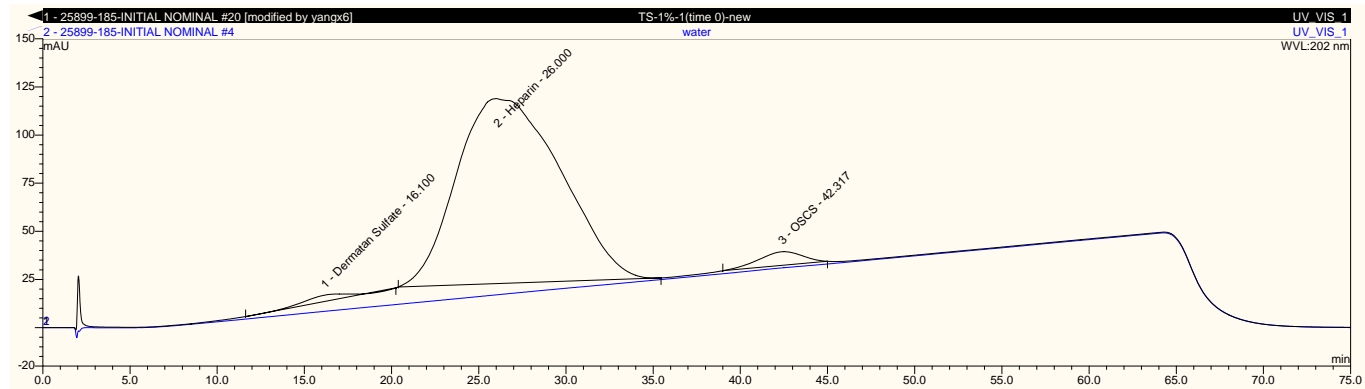
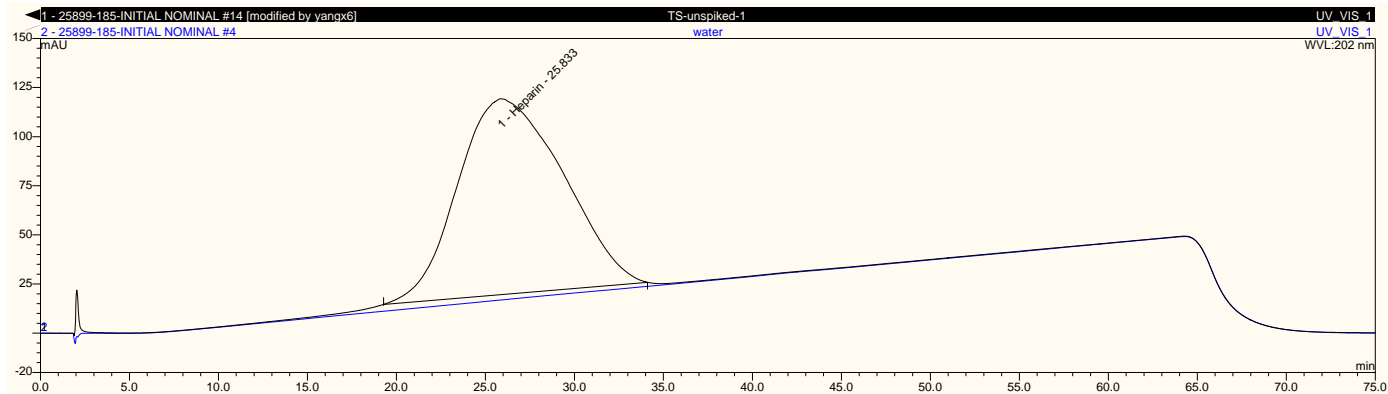
Resolution	USP (DS/Hep)	EP (DS/Hep)	USP(Hep/OSCS)	EP (Hep/OSCS)
SS-1% (black)	0.95, 0.91, 1.00 Mean 0.95	1.05, 1.02, 1.09 Mean 1.06	1.89, 1.80, 1.78 Mean 1.82	1.84, 1.82, 1.83 Mean 1.83
SS-10% (blue)	1.11, 1.13, 1.18 Mean 1.14	1.15, 1.10, 1.14 Mean 1.13	1.80, 1.96, 1.88 Mean 1.88	1.85, 1.90, 1.85 Mean 1.87

Heparin Chromatographic Identification

Example chromatograms for analysis of Heparin Sodium Raw Material (20 mg/mL)

Top: unspiked, overlay with water injection

Bottom: spiked with 1% DS and 1% OSCS, overlay with water injection

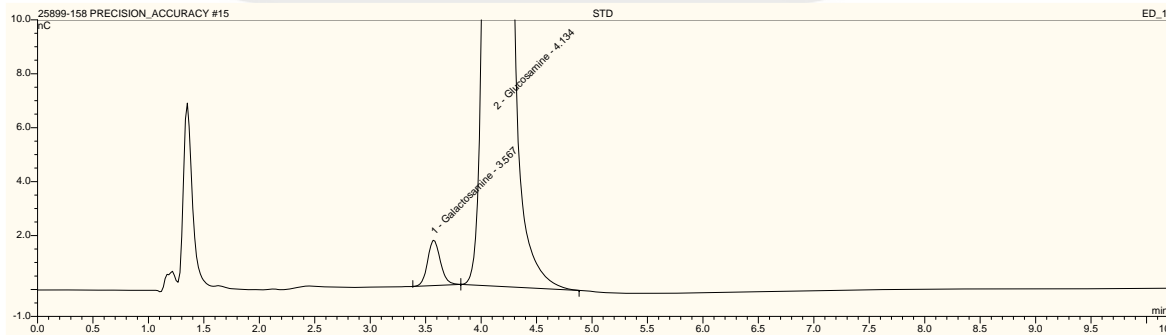


Lessons Learned:

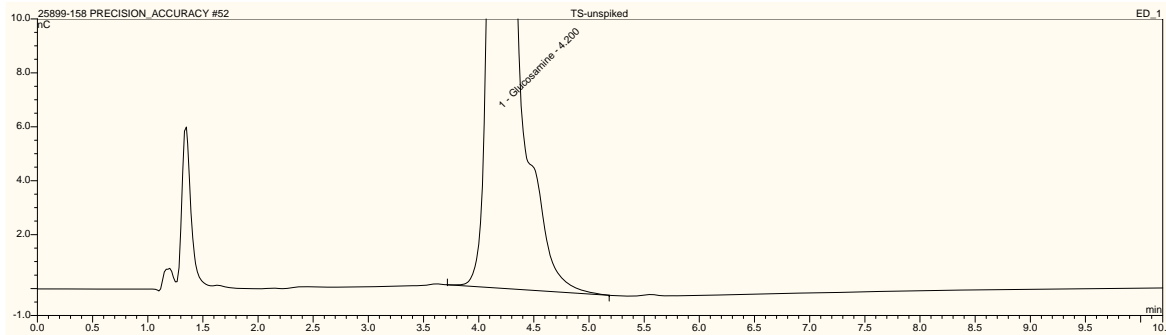
- Mobile phase was validated with $\text{NaH}_2\text{PO}_4 \cdot 2 \text{H}_2\text{O}$ and $\text{NaClO}_4 \cdot \text{H}_2\text{O}$
- Column temperature should be 40°C to achieve the retention times for dermatan, heparin, and OSCS stated in the procedure.
- Waters system with Empower gives better resolution than Dionex with Chromeleon software due to Empower selection of peaks.
- Half height method for calculating resolution (Chromeleon default method) should be used for asymmetric peaks.
- Potential increase in dermatan content (10%) may improve resolution.

Brief description of the proposed method

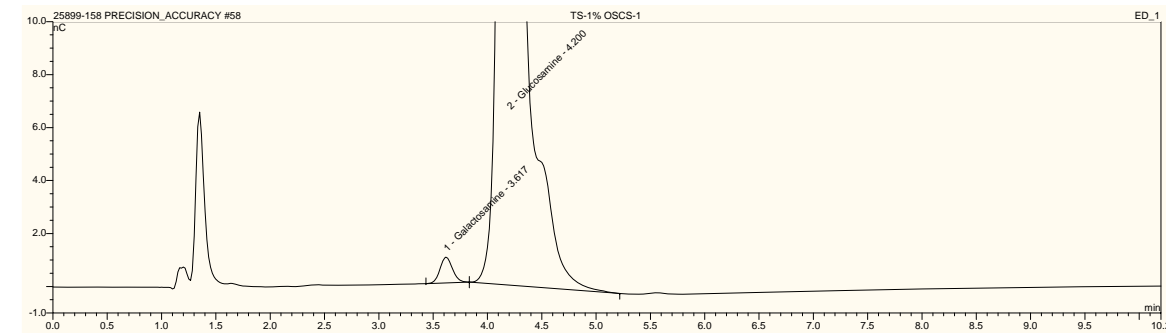
- Instrument used: Dionex ICS 3000 with Chromeleon software
- Columns: Dionex CarboPac PA20, 3 X 30 mm guard, BioLC Amion Trap, 3 X 30 mm, and PA20, 3 X 150 mm analytical
- Eluent: 12 mM KOH
- Flow rate: 0.5 mL/min, Injection volume: 10 μ L, column temp: 30°C
- Pulsed amperometric detection
- Standards and samples in 5 N HCl are hydrolyzed for 6 hours at 100°C
- Runtime: 35 min (including column cleaning and equilibration)
- Resolution: NLT 2 between galactosamine and glucosamine peaks
- NLT 2,000 theoretical plates for glucosamine and tailing factor between 0.8 and 2.0 for the galactosamine and glucosamine peaks
- Acceptance criterion: The percent of galactosamine of the total hexosamine of the hydrolyzed sample solution must be NMT 1%



Standard Solution



Heparin Sodium raw material



Heparin Sodium raw material spiked with 1% OSCS :upon hydrolysis, OSCS is converted to galactosamine

Only amino trap and analytical columns used for all above injections.

Lessons Learned:

- Water determination of galactosamine HCl USP Reference Standard
 - Coulometric Karl Fisher method used due to the water content less than 1%.
 - Solubility problem with the commonly used KF reagents, potential side reaction going on and difficult to get stable endpoint.
 - Oven will be used to purge water into the reaction vessel for Karl Fisher water analysis to avoid side reaction with galactosamine.

- We recommend using analytical column with only amino trap column:
 - No incremental gain in chromatographic attributes (e.g. resolution) when a guard column is also used; rather there is a loss in theoretical plates and the shape of glucosamine peak.
 - Dionex does not recommend using both of these protective columns.
 - Troubleshooting in a quality control laboratory would be a lot easier when only one of the protective columns is used.

- “Acceptance criteria: The percent galactosamine peak area of the total hexosamine of the *Hydrolyzed sample solution* must be NMT 1%” (quote from the proposal).
 - Should be the weight percentage of galactosamine of total hexosamine instead of peak area percentage, since the response ratio of galactosamine to glucosamine is used.

Lowry Method

- Protein reacts with Alkaline Cupric Sulfate in the presence of tartrate during a 10 minute incubation.
- A tetradentate copper complex forms from four peptide bonds and one atom of copper. The complex is light blue in color
- The Folin phenol reagent (phosphomolybdic/phosphotungstic acid complex) is added and is reduced when the copper complex transfers electrons to the Folin phenol reagent.
- The reduced Folin phenol reagent is bright blue and the color intensifies during a 30 minute reaction.

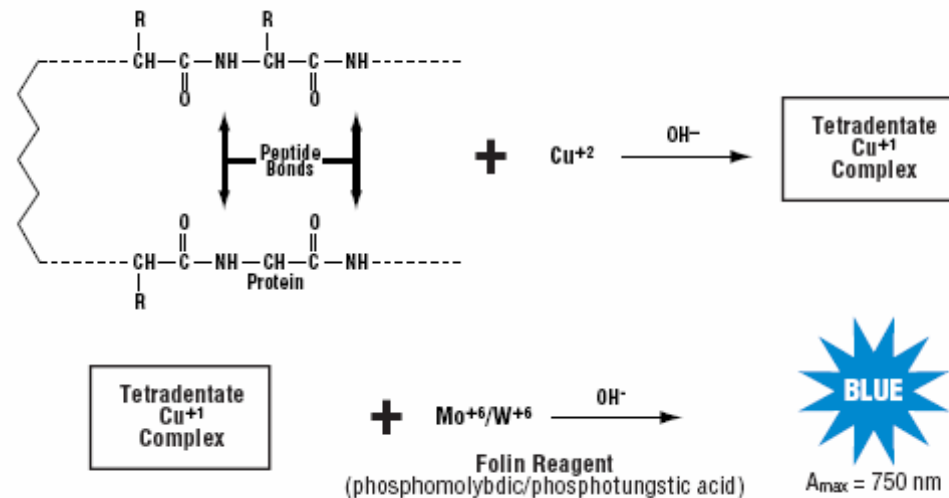


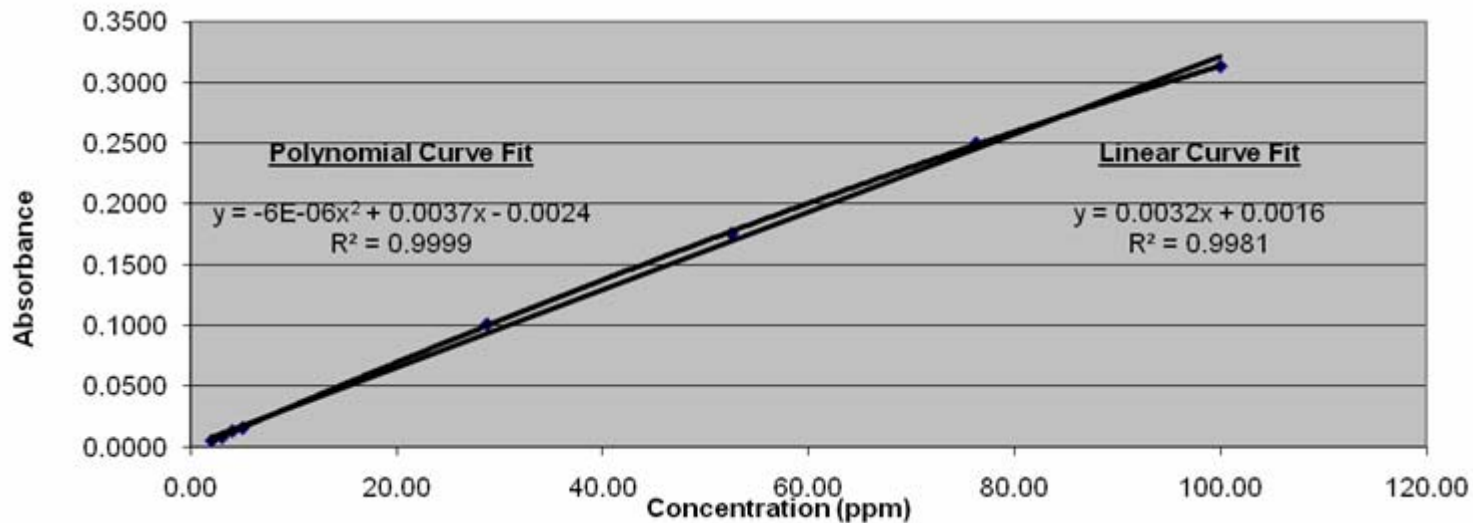
Image from Pierce Guide for Protein Assays

Proposed Monograph PF 35(2) [Mar-Apr 2009]

- Standard Curve - 0.0016 to 0.032 mg/mL BSA
- Sample Solution – 30 mg/mL Heparin Sodium in water
- Specification NMT 1%
- In evaluation of multiple suppliers and lots of API, obtained results ranging from 0.04 to 0.2% Protein

Revision to Proposed Monograph PF Vol 35(2)

- Standard Curve - 0.005 to 0.100 mg/mL BSA
- Standard Solution – 5 mg/mL Heparin Sodium in Water



Interference in the Lowry Assay

- Heparin Documented as an Interfering Substance
 - Peterson, G.L. (1982). Meth. In Enzymol. Hirsch C.H.W. and Timasgeff S. N., eds. San Diego: Academic Press, 91, pp. 95-119.
- In laboratory Studies OSCS and Dermatol were also demonstrated to interfere with the protein assay

Evaluated Interfering Substances Preparation

- Followed <1057> Interfering Substances Procedure
- In evaluation of the sample lots of API, no protein was detected
- Recovery Studies using API samples spiked with 0.05% protein resulted in 105 - 106% recovery

Proposed Changes to determine the effect on interference

- In samples from multiple API suppliers run without interfering substances procedure:
 - Results ranged from 0.02 to 0.2% Protein
- In the same samples run with the interfering substances procedure:
 - No protein was detected
- With both procedures spike recoveries were 105-108%

Conclusion

- Even with concentration change, the option of the interfering substances preparation may still be necessary.

Heparin Interference with Protein Assay

Preparation of Folin's Phenol Reagent

“Diluted Folin-Ciocalteu's Phenol reagent: Dilute Folin-Ciocalteu's Phenol reagent 2–4 times with water. The dilution should be chosen such that the pH of the Samples (i.e. Standard and sample solutions after addition of Lowry reagent C and the diluted Folin-Ciocalteu's Phenol reagent) is 10.25 ± 0.25 .”

Folins Reagent was diluted 2X for experiments below.

- The amount of Folins present affects absorbance and so it must be the same for samples and standards.
- Increased dilution of the Folins will reduce the sensitivity.

Interfering Substances preparation resulted in sample at the pH limit.

- When pH was checked prior to 30 minute incubation, the pH exceeded 10.50

Sample	pH				pH
	Kit Lowry	USP Lowry	IS Kit Lowry	IS USP Lowry	
Kit Lowry					12.75
USP Lowry					12.76
Diluted Folins					0.82
API A					6.67
API B					6.66
50 ppm BSA					6.59
API A + Lowry immediate	12.65	12.69	12.69	12.75	
API B + Lowry immediate	12.72	12.68	12.70	12.74	
50 ppm BSA + Lowry immediate	12.64	12.69	12.70	12.75	
API A + Lowry + Folins NLT 30 min.	10.27	10.26	10.41	10.54	
API B + Lowry + Folins NLT 30 min.	10.41	10.41	10.38	10.50	
50 ppm BSA + Lowry + Folins NLT 30 min.	10.27	10.41	10.39	10.53	

IS = Interfering Substances Preparation

Proposed Changes to Draft Procedure:

- Standard curve range should be changed to 0.02 to 0.10 mg/mL and the heparin sample concentration changed to 5 mg/mL.
- Interfering substances procedure should be used since heparin is a known interfering substance for Lowry protein method.
- Do not introduce the pH criteria since there are no known interpretations of pH for Lowry method outside of range of 10.25 ± 0.25 .

Testing for Impurities in Heparin Scorecard

Identity and Organic Impurity Methods

Potential Impurities	NMR	Chromatographic Identity	Anti-factor Xa / anti-factor IIa	Sodium (flame)	Galactosamine in total hexosamine	Nucleotides	Protein (Lowry)	Residual Solvents
Residual protein-carbohydrate linker	Green				Green			
Dermatan		Yellow			Green			
Chondroitin					Green			
Process oxidation-peroxidation by-products	Green							
Residual solvents	Yellow							Green
Residual protein							Green	
Residual nucleic acids						Yellow		
OSCS	Green	Yellow			Green			

Testing for Impurities in Heparin Scorecard

Identity and Organic Impurity Methods

Potential Impurities	NMR	Chromatographic Identity	Anti-factor Xa / anti-factor IIa	Sodium (flame)	Galactosamine in total hexosamine	Nucleotides	Protein (Lowry)	Residual Solvents	Improved CE
Residual protein-carbohydrate linker	Green				Green				
Dermatan		Yellow			Green				Green
Chondroitin					Green				Green
Process oxidation-peroxidation by-products	Green								?
Residual solvents	Yellow							Green	
Residual protein							Green		
Residual nucleic acids						Yellow			
OSCS	Green	Yellow			Green				Green

- Impurity testing methods have significantly improved
 - prior to June 2008
 - phase I methods (NMR and CE)
 - phase II methods (enhanced NMR and more specific methods)
- Proposed methods for Chromatographic Identity and Protein Impurity need further improvements to robustness and accuracy.
- Additional method to test charge and molecular weight would further enhance the testing scheme.

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