Application note

Anti-Factor Xa and IIa Assays for Unfractioned Heparin Activity:

Spectrophotometric Analysis According to USP Monograph

Introduction

Heparin, a polysaccharide compound, is an anti-coagulant used to treat thromboembolisms and other conditions.[1-3] The compound is often administered prior to kidney dialysis.[4] Heparin functions as it does due to a particular pentasaccharide region that consists of sulfated glucosamine residues not commonly found elsewhere in the compound. This pentasaccharide region will bind with antithrombin, a protein in the blood stream which functions as a naturally occurring mild blood thinner.[5] The resulting heparin-antithrombin complex inhibits serine proteases such as factor IIa and factor Xa at much greater rates than antithrombin alone does. These proteases, factor IIa and factor Xa, are enzymes which aid in coagulation, and their inhibition by the heparin-antithrombin complex (known as anti-factor IIa and anti-factor Xa activity), prevents blood coagulation.[1, 2]

To ensure the drugs used in clinical environments behave as expected and do not produce unwanted side effects, it is important the quality of the product is verified prior to distribution. For example, some batches of manufactured heparin have been found to be contaminated by the presence of oversulfated chondroitin sulfate.[4] The presence of this moiety in the produced drug has been linked to allergic reactions. Adverse effects like this highlight the need to thoroughly test the quality of drug products prior to distribution. For manufactured heparin, it is vital that the activity of the heparin-antithrombin complex meets designated standards to ensure the quality of the final product.

The US Pharmacopeia (USP) has developed a quality test to ensure the activity of the manufactured heparin meets required specifications.[6] In this method, the activities of the produced heparin and of USP heparin standards for both factor IIa and factor Xa are determined using a colorimetric assay. This assay requires the use of a UV-Visible absorption spectrophotometer. The test compares the measured absorbance of a set of heparin sample solutions to a set of standard solutions. The ratio of the activity of the heparin samples versus the activity of the standards, for both anti-factor IIa and anti-factor Xa, is then compared to acceptance criteria.

Herein, the Thermo Scientific™ Evolution™ One Plus UV-Vis Spectrophotometer was used to measure the absorption of the prepared heparin standards and samples in the presence of a chromogenic substrate. This instrument meets USP requirements outlined in USP <857>.[7] The sample preparation and measurements were carried out based on USP monograph <208>. Data analysis was performed using the built-in analysis functionalities in the instrument software.

Experimental

Anti-factor IIa Assay

The procedure known as the anti-factor IIa assay was carried out based on the USP <208> procedure.[6] (The method described herein deals with unfractionated heparin; the same USP procedure includes a description for analyzing low molecular weight heparin as well but that is not considered here.)

Briefly, solutions of heparin USP standards at 4 different concentrations (0.030, 0.020, 0.010 and 0.005 IU/mL) were prepared by diluting a 2.1 IU/mL solution of USP standard heparin sodium salt with a buffer solution (50 mM tris(hydroxymethyl)aminomethane, 175 mM NaOH in pH 7.6 tris-ethylenediaminetetraacetic acid, and 0.1% polyethylene glycol 6000 in deionized water). 100 µL aliquots of the resulting standards were then incubated in a conical vial at 37 °C for 15 min using a heating block. A "blank" sample consisting of only the buffer solution was also incubated following the same procedure.

Stock solutions of antithrombin (AT, 0.125 IU/mL), and factor IIa, thrombin (T, 5.0 U/mL), were made by reconstituting the purchased material with the buffer solution described previously. A stock solution of 1.25 mM S-2238, the chromogenic dye, was prepared using de-ionized (DI) water as the solvent. The 20% (v/v) acetic acid (AA) stock solution was made by diluting 1.0 mL of glacial acetic acid with 4.0 mL of DI water. The various stock solutions (AT, T, S-2238 and AA) were then incubated for 15 min at 37 °C using a heating block.

Aliquots of the stock solutions were added to each standard and blank solution, over time and in this order: 200 µL of 0.125 IU/mL AT, 50 µL of 5.0 U/mL T, 100 µL of 1.25 mM S-2238, and 100 µL 20% (v/v) AA. In between each addition, the solution was incubated at 37 °C for 2 min. A solution was also made to zero/blank the spectrophotometers by adding the same components in reverse order to 100 µL of the buffer solution, starting with 20% (v/v) AA and ending with 0.125 IU/mL AT.

A stock solution of heparin was also prepared by dissolving 10.4 mg of heparin sodium salt in 10 mL of the same buffer solution. Sample solutions of heparin were then prepared using this heparin stock solution to achieve 4 different concentrations (0.030, 0.020, 0.010 and 0.005 IU/mL). A separate solution was also made without the addition of heparin. The same procedure as above was followed, except the heparin USP standards were replaced with these stock heparin samples.

Anti-factor Xa Assay

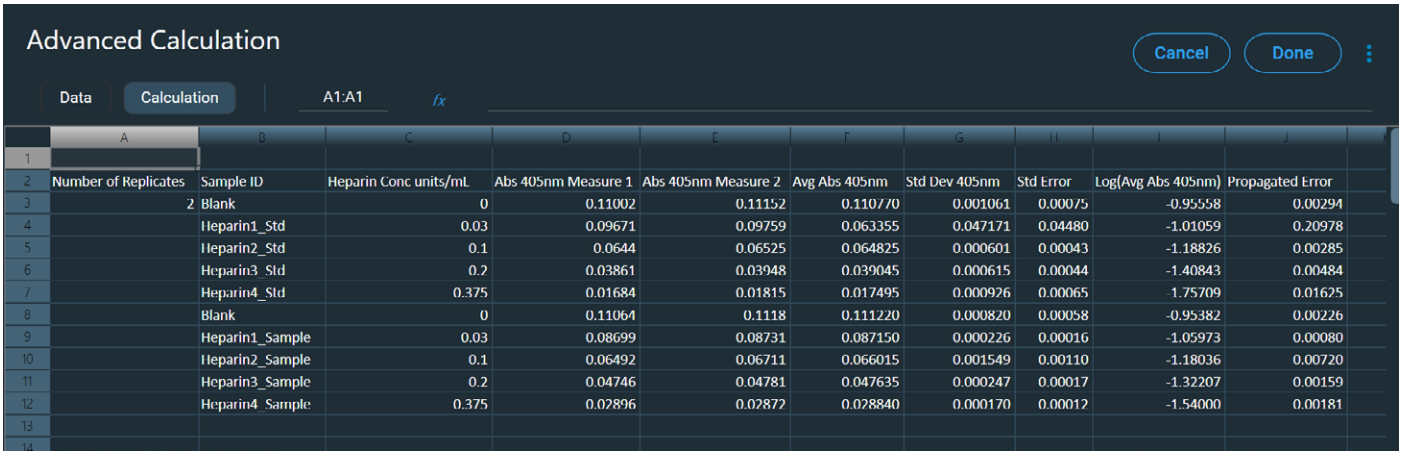
The anti-factor Xa assay procedure to analyze unfractioned heparin was carried out based on the same USP <208> procedure.[6] (Again, the USP procedure includes a description for analyzing low molecular weight heparin as well but is not utilized here.) Standard solutions and sample solutions were prepared by diluting the respective heparin stock solutions with the buffer solution, resulting in solution concentrations of 0.03, 0.1, 0.2, and 0.35 IU/mL. A solution containing no heparin was also made. 30 µL of each standard and sample solution, as well as the buffer solution containing no heparin, were incubated at 37 °C for 15 min in a conical vial.

Similar to the anti-factor IIa assay, stock solutions of AT (1.0 IU/mL), and factor Xa (Xa, 3.0 U/mL) were made by reconstituting the material with the buffer solution previously described. A 1.0 mM S-2222 chromogenic dye stock solution was also made in DI water. The same 20% (v/v) AA stock solution was used as is described for the anti-factor IIa assay.

To each 30 µL aliquot of the sample, standard and blank solutions, the following solutions were added over time and in this order: 150 µL of AT (1.0 IU/mL), 300 µL of Xa (3.0 U/mL), 300 µL of S-2222 (1.0 mM), and 150 µL of AA (20% v/v). The reaction mixture was incubated at 37 °C for 2 min in between each addition step. The zero solution was made by adding each component to 100 mL of the buffer solution in reverse order, starting with 20% (v/v) AA and ending with 1.0 IU/mL AT.

UV-Visible Absorption Measurements

The absorbance of the standards and samples for both anti-factor IIa and Xa assays was measured at 405 nm using a Thermo Scientific™ Evolution™ One Plus Spectrophotometer. The spectral bandwidth and integration time were set to 1.0 nm and 1.0 s, respectively. As per USP's procedure, measurements were performed back-to-back in the following order to obtain duplicate measurements: blank 1, standard solutions, blank 2, sample solutions, blank 2, samples solutions, blank 1, standard solutions. The zero solution was used to collect the blank measurement prior to measuring the standards and samples for the respective assay.



	A	B	C	D	E	F	G	H	I	J
1										
2	Number of Replicates	Sample ID	Heparin Conc units/mL	Abs 405nm Measure 1	Abs 405nm Measure 2	Avg Abs 405nm	Std Dev 405nm	Std Error	Log(Avg Abs 405nm)	Propagated Error
3	2	Blank	0	0.11002	0.11152	0.110770	0.001061	0.00075	-0.95558	0.00294
4		Heparin1_Std	0.03	0.09671	0.09759	0.063355	0.047171	0.04480	-1.01059	0.20978
5		Heparin2_Std	0.1	0.0644	0.06525	0.064825	0.000601	0.00043	-1.18826	0.00285
6		Heparin3_Std	0.2	0.03861	0.03948	0.039045	0.000615	0.00044	-1.40843	0.00484
7		Heparin4_Std	0.375	0.01684	0.01815	0.017495	0.000926	0.00065	1.75709	0.01625
8		Blank	0	0.11064	0.1118	0.111220	0.000820	0.00058	-0.95382	0.00226
9		Heparin1_Sample	0.03	0.08699	0.08731	0.087150	0.000226	0.00016	-1.05973	0.00080
10		Heparin2_Sample	0.1	0.06492	0.06711	0.066015	0.001549	0.00110	-1.18036	0.00720
11		Heparin3_Sample	0.2	0.04746	0.04781	0.047635	0.000247	0.00017	-1.32207	0.00159
12		Heparin4_Sample	0.375	0.02896	0.02872	0.028840	0.000170	0.00012	-1.54000	0.00181
13										
14										

Figure 1. Advanced calculation window in Insight Pro. Data displayed was collected from the Heparin anti-factor assay samples.

Results/Discussion

Following data collection, the log of the measured absorbance, as well as the measurement error, was calculated using the Advanced Calculation interface in the instrument software (Thermo Scientific™ Insight™ Pro) as shown in Figure 1. The collected data, as well as the calculated values, are reported in Table 1 for the anti-factor IIa experiment and in Table 2 for the anti-factor Xa experiment. The standard deviation in the absorbance of the collected blank solutions was <0.3% of the average value for the standard and sample measurements from the anti-factor IIa assay, and <1.0% for the anti-factor Xa assay. These variations are within the limit (<10%) defined by USP.

Sample Type	Heparin Concentration (IU/mL)	$Abs_{405\ nm}$ Replicate 1	$Abs_{405\ nm}$ Replicate 2	Average Abs ± Standard Error	Log(Abs) ± Standard Error
Standards	0.000	0.14975	0.15029	$0.1500 \pm 2.7 \times 10^{-4}$	$-0.8239 \pm 7.8 \times 10^{-4}$
	0.005	0.1206	0.12071	$0.12066 \pm 5.0 \times 10^{-5}$	$-0.9185 \pm 2.0 \times 10^{-4}$
	0.010	0.07509	0.07547	$0.0753 \pm 1.9 \times 10^{-4}$	$-1.123 \pm 1.1 \times 10^{-3}$
	0.020	0.03506	0.03523	$0.03515 \pm 8.0 \times 10^{-5}$	$-1.454 \pm 1.1 \times 10^{-3}$
	0.030	0.01151	0.01183	$0.0117 \pm 1.6 \times 10^{-4}$	$-1.933 \pm 6.0 \times 10^{-3}$
Samples	0.000	0.15046	0.15057	$0.15052 \pm 5.0 \times 10^{-5}$	$-0.8224 \pm 1.6 \times 10^{-4}$
	0.005	0.1249	0.12505	$0.12498 \pm 7.0 \times 10^{-5}$	$-0.9032 \pm 2.6 \times 10^{-4}$
	0.010	0.08756	0.08798	$0.0878 \pm 2.1 \times 10^{-4}$	$-1.057 \pm 1.0 \times 10^{-3}$
	0.020	0.04241	0.04282	$0.0426 \pm 2.0 \times 10^{-4}$	$-1.370 \pm 2.1 \times 10^{-3}$
	0.030	0.02017	0.02053	$0.0204 \pm 1.8 \times 10^{-4}$	$-1.691 \pm 3.8 \times 10^{-3}$

Table 1. Collected and Calculated Results for Heparin Anti-factor IIa Assay.

Sample Type	Heparin Concentration (IU/mL)	$Abs_{405\ nm}$ Replicate 1	$Abs_{405\ nm}$ Replicate 2	Average Abs ± Standard Error	Log(Abs) ± Standard Error
Standards	0.000	0.11002	0.11152	$0.1108 \pm 7.5 \times 10^{-4}$	$-0.956 \pm 2.9 \times 10^{-3}$
	0.030	0.09671	0.09759	$0.0972 \pm 4.4 \times 10^{-4}$	$-1.013 \pm 2.0 \times 10^{-3}$
	0.100	0.0644	0.06525	$0.0648 \pm 4.3 \times 10^{-4}$	$-1.188\ 2.9 \times 10^{-3}$
	0.200	0.03861	0.03948	$0.0390 \pm 4.4 \times 10^{-4}$	$-1.408 \pm 4.8 \times 10^{-3}$
	0.375	0.01684	0.01815	$0.0175 \pm 6.5 \times 10^{-4}$	$-1.76 \pm 1.6 \times 10^{-2}$
Samples	0.000	0.11064	0.1118	$0.1112 \pm 5.8 \times 10^{-4}$	$-0.954 \pm 2.3 \times 10^{-3}$
	0.030	0.08699	0.08731	$0.0872 \pm 1.6 \times 10^{-4}$	$-1.0597 \pm 8.0 \times 10^{-4}$
	0.100	0.06492	0.06711	$0.066 \pm 1.1 \times 10^{-3}$	$-1.180 \pm 7.2 \times 10^{-3}$
	0.200	0.04746	0.04781	$0.0476 \pm 1.7 \times 10^{-4}$	$-1.322 \pm 1.6 \times 10^{-3}$
	0.375	0.02896	0.02872	$0.0288 \pm 1.2 \times 10^{-4}$	$-1.540 \pm 1.8 \times 10^{-3}$

Table 2. Collected and Calculated Results for Heparin Anti-factor Xa Assay.

The slope-ratio analysis method was used to further work up the data. This involved plotting the log of the average absorption of a given sample or standard against the sample/standard's heparin concentration (Figure 2) and fitting the resultant plot to a linear function. The slope of the best fit line, as well as the R^2 values, are summarized in Table 3. Given the R^2 values are close to 1, the data depicted in Figure 2 fits well to a line, indicating the slope-ratio assay statistical method is appropriate to use for this analysis.

Assay		Slope (mL/IU)	R^2
Anti-Factor Xa	Standard	-2.16	0.998
	Sample	-1.50	0.980
Anti-Factor IIa	Standard	-37.3	0.987
	Sample	-29.8	0.994

Table 3. Calculated slope and R^2 from the linear fit functions in Figure 2. Data was analyzed using the slope ratio assay method.

Following fitting the data to a line, the activity of the heparin in the sample solutions was then determined through equation 1,

$$P_{Sample} = P_{Std}\left(\frac{m_{Sample}}{m_{Std}}\right)$$

Equation 1.

Where P_{Sample} is the calculated activity of heparin in the samples, P_{Std} is the activity of the USP standard (180 units/mg), m_{Sample} is the slope of the linear fit for the samples (Figure 2) and m_{Std} is the slope of the linear fit for the USP standard solutions (Figure 2). Using equation 1, the activity for heparin as determined in the anti-factor IIa assay was found to be 144 units/mg, while the activity was found to be 125 units/mg when using the anti-factor Xa assay.

For the anti-factor IIa assay, USP states the activity should be no less than 180 units/mg. The measured value of 144 units/mg fell well below that, indicating the samples described herein failed this test. This may have been a result of age of the heparin used, or the storage conditions of the material.

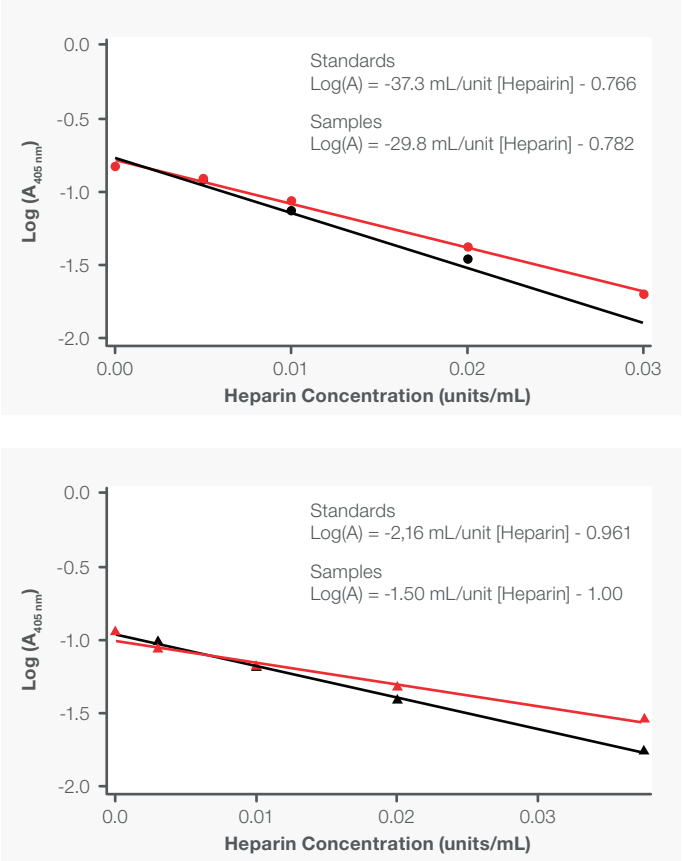


Figure 2. Log of the average absorbance collected at 405 nm for (a) anti-factor IIa and (b) anti-factor Xa assays as a function of heparin concentration (units/mL). Samples were prepared using USP standard Heparin (black) and a separate heparin source (red). Error bars are included for each data point.

To assess the overall activity of the heparin for inhibition of both factor IIa and Xa, the ratio between the activity (equation 2) calculated from both assays using equation 1 was followed.

$$\frac{P_{sample,anti\text{-}factor\ Xa}}{P_{sample,anti\text{-}factor\ IIa}}$$

Equation 2.

According to USP, this value should fall between 0.9 and 1.1 for unfractioned heparin.[6] For the samples measured herein, the ratio was found to be 0.87, which is outside of the acceptable range. As described earlier, this discrepancy may be due to the age of the heparin samples studied or the storage conditions of the sample.

Conclusions

The analysis of heparin anti-factor IIa and Xa activity was carried out based on the relevant USP monograph. This method, used to determine the activity of heparin for inhibition of factor IIa and Xa, required the use of a UV-Vis absorption spectrophotometer and subsequent data workup. Through the Advanced Calculation functionality in the instrument software, the slope-ratio assay analysis was carried out prior to data exportation, limiting the time needed to assess the quality of the manufactured heparin. Herein, the resulting activity of the sample heparin was found to be 144 units/mg and 125 units/mg for the anti-factor IIa and Xa assays, respectively, compared to the activity of the USP standard Heparin (180 units/mg). The ratio of these values was found to be 0.87, outside of the acceptable range. The UV-Vis spectrophotometer was useful in confirming that the particular heparin samples analyzed in this experiment did not meet established USP standards.

References

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