

which the substrate is an oligopeptide of a specific structure with an attached chromogen (para-nitroaniline, pNA). Factor Xa, a serine protease, releases the chromogen which can be quantitated spectrophotometrically at 405 nm. The principle of the anti-Xa activity assay is based on the inhibitory effect of enoxaparin-antithrombin III-complex on the hydrolytic activity of factor Xa towards its specific substrate. As an accurately defined amount of factor Xa is added to the incubation medium, a proportional relationship exists between the absorbance recorded at 405 nm and the enoxaparin-antithrombin complex. The specific oligomer substrate used is CBS 3139.

Control samples consisting of matrix blank urine from healthy volunteers were spiked with known amounts of enoxaparin spanning a range of 0.025 to 0.800 IU anti-Xa /mL and were assayed for intra-assay evaluation. Four urine samples with 0.025 (2 samples) and 0.800 (two samples) IU anti-Xa/mL were included in each assay system to estimate inter-assay precision.

After thawing, the urine samples (2mL) were mixed with 5 mL absolute alcohol and centrifuged at 3000 rpm at room temperature. The precipitate was dissolved in 0.5 mL NaCl, mixed and ultra-sonicated for 2 min. Subsequently 1.5 mL NaCl was added and the extraction procedure repeated.

Each urine extract was diluted 10 fold with antithrombin III solution. The specific anti-Xa activity was determined with the chromogenic substrate CBS 3139 and bovine factor Xa. 0.1 mL urine extract were mixed with 0.050 mL factor Xa and incubated 90 sec at 37° C. Then 0.075 mL of CBS 3139 was added and the increase in absorbance at 405 nm during 60 seconds recorded.

Standard calibration curves were constructed using activities of 0, 0.025, 0.05, 0.1, 0.2, 0.4, 0.6 and 0.8 IU anti-Xa/mL.

Results

The calibration curves obtained were linear over the entire range of activities tested.

The results on the intra- and inter-assay accuracy and precision are shown in Tables 6 and 8:

Table n°5 : Spikes - Intra-assay reproducibility in urine
Values of optical density

Standard curve n°	Theoretical activity (IU aXa/ml)						
	0.025	0.050	0.100	0.200	0.400	0.600	0.800
1	0.824	0.794	0.781	0.722	0.604	0.523	0.465
2	0.809	0.796	0.761	0.702	0.597	0.515	0.454
3	0.821	0.776	0.765	0.712	0.597	0.526	0.454
4	0.827	0.808	0.780	0.715	0.602	0.520	0.451
5	0.805	0.806	0.772	0.708	0.600	0.520	0.459
6	0.822	0.804	0.765	0.711	0.603	0.519	0.445
7	0.829	0.813	0.787	0.716	0.604	0.520	0.435
8	0.827	0.779	0.767	0.711	0.605	0.530	0.477
Mean	0.821	0.797	0.772	0.712	0.602	0.522	0.455
S.D.	0.009	0.014	0.009	0.006	0.003	0.005	0.013
C.V. %	1	2	1	1	1	1	3

Table n°6 : Spikes - Intra-assay reproducibility in urine
Computed activities of each data related to each calibration curve

Standard curve n°	Theoretical activity (IU aXa/ml)						
	0.025	0.050	0.100	0.200	0.400	0.600	0.800
1	0.028	0.079	0.100	0.207	0.449	0.644	0.803
2	0.041	0.063	0.124	0.232	0.450	0.648	0.817
3	0.026	0.098	0.116	0.210	0.433	0.595	0.783
4	0.021	0.052	0.098	0.212	0.437	0.619	0.815
5	0.047	0.045	0.101	0.213	0.428	0.614	0.776
6	0.027	0.057	0.124	0.223	0.445	0.647	0.854
7	0.031	0.057	0.101	0.227	0.454	0.654	0.892
8	0.005	0.086	0.107	0.209	0.428	0.607	0.749
Mean	0.028	0.067	0.109	0.217	0.441	0.629	0.811
S.D.	0.013	0.018	0.011	0.009	0.010	0.022	0.045
C.V. %	45	28	10	4	2	4	6
Difference %	13	34	9	8	10	5	1

Table n°7 : Spikes - inter-assay reproducibility in urine
Values of optical density

Standard curve n°	Theoretical activity (IUaXa/ml)			
	0.025	0.025	0.800	0.800
1	0.822	0.830	0.410	0.416
2	0.816	0.823	0.493	0.486
3	0.806	0.813	0.404	0.407
4	0.800	0.809	0.392	0.389
5	0.794	0.803	0.393	0.392
6	0.806	0.807	0.390	0.396
7	0.817	0.820	0.491	0.488
8	0.816	0.817	0.471	0.474
9	0.803	0.808	0.395	0.394
10	0.811	0.810	0.393	0.395
Mean	0.809	0.814	0.423	0.424
SD	0.009	0.008	0.043	0.042
C.V. %	1	1	10	10

Table n°8 : Spikes - inter-assay reproducibility in urine
Computed activities of each data related to individual calibration curve

Standard curve n°	Theoretical activity (IUaXa/ml)			
	0.025	0.025	0.800	0.800
1	0.029	0.018	0.815	0.798
2	0.033	0.020	0.784	0.805
3	0.038	0.028	0.817	0.808
4	0.040	0.028	0.775	0.782
5	0.037	0.025	0.790	0.792
6	0.030	0.029	0.824	0.808
7	0.041	0.036	0.805	0.814
8	0.038	0.036	0.735	0.727
9	0.033	0.027	0.810	0.812
10	0.035	0.036	0.821	0.816
Mean	0.035	0.028	0.798	0.796
SD	0.004	0.006	0.027	0.027
C.V. %	12	23	3	3
Difference %	41.60	13.20	-0.30	-0.48

The intra-assay and inter-assay accuracies and precision for the 0.025 IU anti-Xa level in the QC samples exceeded the allowable limit of 20%.

The baseline values determined for endogenous mucopolysaccharides in urine ranged between 0.044 IU anti-Xa/mL (1 female subject) to 0.168 IU anti-Xa/mL (1 male subject). Setting up the standard curves in each volunteer's urine minimizes the impact of endogenous mucopolysaccharides on the quantitation of enoxaparin.

Conclusion

The assay based on the anti-Xa activity of enoxaparin in urine can only measure anti-Xa activity of 0.800 IU anti-Xa/mL. Information on the stability of the anti-Xa activity in urine is lacking and thus the assay cannot be considered validated.

Comments

1. The validation report for the measurement of anti-Xa activity in urine does not address stability issues including impact of storage and freeze and thaw cycles and exposure of the samples to room temperature. The assay cannot be considered validated. The sponsor appears to agree with that assessment stating that "the assay may be applied to a preliminary estimation of biological active compounds excreted in urine".

Anti-IIa Activity of Enoxaparin in Plasma

Study _____ Anti-IIa Chromogenic Assay Supplemented with Antithrombin III for the Measurement of Enoxaparin in Human Plasma

Study Investigator and Study Site: _____

b(4)

b(6)

Objectives

A specific amidolytic method assay with antithrombin III supplementation is described that measures the anti-IIA activity of enoxaparin in plasma. Supplementation of antithrombin reduces the influence of varying concentrations of endogenous antithrombin.

Methods

The principle of the amidolytic assay is based on the inhibitory effect of the enoxaparin-antithrombin III complex on the hydrolytic capacity of Factor IIa (thrombin) to hydrolyze a specific synthetic substrate, S 2238. An accurately measured amount of Factor IIa is

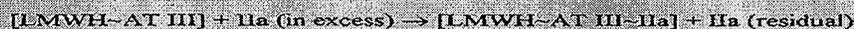
added to the incubation medium after addition of antithrombin III. An additional problem with determining reliable anti-IIa activities *ex vivo* is the confounding impact of PF4 (platelet derived heparin neutralizing protein). Enoxaparin and other low molecular weight heparins (LMWH) are as sensitive as un-fractionated heparin regarding neutralization of their antithrombin activities by the impact of PF4. Details of the assay method are described below:

The anti-IIa supplemented with AT III assay is a two-stage anti-IIa amidolytic assay developed according to TEIEN and LIE [9]. The principle is based on the inhibitory effect of the [LMWH-AT III] complex on the hydrolytic capacities of the factor IIa toward its specific synthetic substrate. The thrombin is added to the incubation medium after dilution with AT III solution in an accurately defined amount. The assay is carried out according to the following reactions:

- dilution of the plasma test with the AT III solution:



- then incubation of the solution with a known excess of purified serine protease:



- the residual factor IIa hydrolyses the chromogenic substrate (peptide-pNA) releasing paranitroaniline (pNA):



The quantity of paranitroaniline (pNA free) released is measured spectrophotometrically at 405 nm during a fixed time (initial rate method). The quantity of paranitroaniline released is inversely proportional to the amount of LMWH present in the plasma.

The plot of the logarithm of the absorbance versus the anti-IIa activity of standards produces a linear dose-response curve in the 0.010- 0.150 IU anti-IIa/ml activity range.

The chromogenic substrate used is S 2238. The standard calibration curves had nominal anti-IIa activities of 0.010, 0.020, 0.040, 0.060, 0.080, 0.10, and 0.150 IU/mL. The impact of diluting samples whose activities were expected to be outside of the calibration range was studied by diluting samples with nominal activities of 0.40, 0.30, 0.20 and 0.10 IU anti-IIa/mL activities by factors of 1/3 to 1/7.

Results

There is an inverse relationship between the logarithm of the delta of absorbance at 405 nm and the amount of enoxaparin in human plasma.

Based on precision ($\leq 20\%$) and accuracy ($\leq 20\%$) determinations the LLOQ was set at 0.020 IU anti-IIa/mL.

The within-assay and between assay-accuracies are shown in Tables 5 and 13, respectively:

Table 5 : Within-assay reproducibility of quality controls
Computed activities of each Δ A405nm related to each calibration curve (March 15, 1994)

File name	Nominal activities (IU anti-IIa/ml)						
	QC1A=0.010	QC2A=0.020	QC3A=0.040	QC4A=0.060	QC5A=0.080	QC6A=0.100	QC7A=0.150
Ila15A	0.013	0.017	0.034	0.054	0.071	0.094	0.135
Ila15B	0.013	0.017	0.033	0.052	0.073	0.093	0.143
Ila15C	0.013	0.020	0.034	0.054	0.074	0.091	0.130
Ila15D	0.015	0.021	0.035	0.058	0.076	0.094	0.140
Ila15E	0.015	0.017	0.034	0.052	0.070	0.092	0.142
Ila15F	0.014	0.019	0.031	0.052	0.077	0.090	0.135
Ila15H	0.011	0.020	0.030	0.057	0.068	0.096	0.150
Ila15I	0.009	0.020	0.034	0.051	0.072	0.095	0.143
Ila15J	0.016	0.019	0.036	0.052	0.075	0.100	0.146
Ila15K	0.014	0.017	0.034	0.049	0.070	0.096	0.160
Mean	0.013	0.019	0.034	0.053	0.073	0.094	0.142
SD	0.002	0.002	0.002	0.003	0.003	0.003	0.009
% CV	15	8	5	5	4	3	6
n	10	10	10	10	10	10	10
Min	0.009	0.017	0.030	0.049	0.068	0.090	0.130
Max	0.016	0.021	0.036	0.058	0.077	0.100	0.160
% Difference	33	-6	-16	-12	-9	-6	-5

Table 13 : Between-assay reproducibility of quality controls
Computed activities of each Δ A405nm related to each calibration curve

File name	Nominal activities (IU anti-IIa/ml)						
	QC1=0.010	QC2=0.020	QC3=0.040	QC4=0.060	QC5=0.080	QC6=0.100	QC7=0.150
Ila18B	0.011	0.018	0.036	0.054	0.077	0.099	0.143
Ila22A	0.010	0.015	0.030	0.044	0.063	0.093	0.145
Ila22B	0.008	0.022	0.035	0.053	0.074	0.093	0.151
Ila22C	0.012	0.018	0.037	0.055	0.073	0.096	0.142
Ila22D	0.012	0.023	0.036	0.056	0.073	0.099	0.146
Ila29A	0.006	0.015	0.029	0.055	0.081	0.107	0.146
Ila29B	0.012	0.022	0.034	0.050	0.073	0.088	0.137
Ila29C	0.014	0.020	0.033	0.052	0.076	0.097	0.151
Ila29D	0.014	0.025	0.043	0.057	0.077	0.097	0.137
Ila29E	0.014	0.021	0.036	0.058	0.081	0.103	0.146
Mean	0.011	0.020	0.035	0.053	0.075	0.097	0.144
SD	0.003	0.003	0.004	0.004	0.005	0.005	0.005
% CV	24	17	11	8	7	6	3
n	10	10	10	10	10	10	10
Min	0.006	0.015	0.029	0.044	0.063	0.088	0.137
Max	0.014	0.025	0.043	0.058	0.081	0.107	0.151
% Difference	13	-1	-13	-11	-7	-3	-4

The results indicate that the within-assay and between-assay accuracies and precisions of the QC samples at the 0.010 IU anti-IIa level exceed the allowable limits. Diluting samples by a factor 1/3 to 1/7 did not change accuracy or precision of the assay performance including the linearity of the response.

Conclusion

The accuracy and precision of the anti-II activity based assay at concentrations between 0.020 -0.150 supplemented with exogenous ATIII are within acceptable limits.

Comments

1. The cross-reactivity of Factors IIa and Xa for CBS 3139 and S 2238, respectively, have not been tested. Thus, the specificity of the anti-Xa and anti-IIa assays is not established.

Study: DMPK/FR/2318: 6 Month Stability Data of the Enoxaparin Anti-IIa Activity in Human Plasma Stored at -20° C and -80° C, Addendum to _____ Anti-IIa Chromogenic Assay Supplemented with Antithrombin III for the Measurement of Enoxaparin in Human Plasma”

b(4)

Study Investigator and Study Site: _____

b(6)

Objectives

The report describes the stability of the QC samples spiked with 0.036, 0.073 and 0.140 IU anti-IIa/mL prepared in human plasma from blood collected into Diatube and stored at -20° C or -80° C for up to 6 months.

Methods

The methods used were those described in _____ QC samples with nominal anti-IIa activities of 0.036, 0.073 and 0.140 IU/mL were prepared and stored at -20 °C or -80 °C for 1 week, 2 weeks, 4 weeks, 6 weeks, 2 months, 3 months and 6 months of storage. The anti-IIa activity of the stored QC samples was calculated relative to freshly prepared calibration curves.

b(4)

Results

The results on accuracy and precision of freshly prepared QC samples with nominal anti-IIa activities of 0.040, 0.060 and 0.140 IU anti-IIa/mL shown in Table 3:

Table 3 - Back-calculated activities (IU anti-IIa/mL) of quality controls

Date of assay	Nominal activity (IU anti-IIa/mL)		
	0.040	0.060	0.140
30-Mar-99	0.041	0.062	0.133
6-Apr-99	0.041	0.065	0.172
13-Apr-99	0.041	0.069	0.148
27-Apr-99	0.041	0.061	0.133
11-May-99	0.037	0.065	0.141
1-Jun-99	0.038	0.061	0.138
29-Jun-99	0.040	0.061	0.146
28-Sep-99	0.037	0.061	0.139
Mean	0.039	0.063	0.144
SD	0.002	0.003	0.012
CV%	4.7	4.6	8.7
n	8	8	8
Min	0.037	0.061	0.133
Max	0.041	0.069	0.172
% Diff	-2.2	4.7	2.6

Accuracy ranged between -2.2 % and 4.7% and precision was < 8.7 %.

The results of the stability experiments with QC samples containing nominal anti-IIa activities of 0.036, 0.073 and 0.140 IU anti-IIa/mL at -20° C are shown in Table 4:

Table 4 - Stability data at -20°C

date of assay	Time of storage	Nominal activities (IU anti-IIa/mL)		
		0.036	0.073	0.140
30 Mar 99	T0	0.032	0.073	0.134
		0.035	0.074	0.134
		0.040	0.081	0.148
	Mean	0.036	0.076	0.139
% Diff	-9.3	4.1	-1.0	
06 Apr 99	1 week	0.031	0.060	0.128
		0.032	0.066	0.130
		0.035	0.072	0.145
	Mean	0.033	0.066	0.134
% Diff	-9.3	-9.6	-4.0	
13 Apr 99	2 weeks	0.034	0.073	0.142
		0.037	0.072	0.141
		0.035	0.072	0.141
	Mean	0.035	0.072	0.141
% Diff	-1.9	-9.1	1.0	
27 Apr 99	1 month	0.027	0.054	0.107
		0.026	0.054	0.104
		0.029	0.060	0.123
	Mean	0.027	0.056	0.111
% Diff	-24	-23	-20	
11 May 99	6 weeks	0.029	0.060	0.120
		0.027	0.057	0.123
		0.030	0.066	0.132
	Mean	0.029	0.061	0.125
% Diff	-20	-16	-11	
01 Jun 99	2 months	0.029	0.062	0.124
		0.028	0.058	0.116
		0.027	0.063	0.122
	Mean	0.028	0.061	0.121
% Diff	-22	-16	-14	
29 Jun 99	3 months	0.029	0.063	0.130
		0.031	0.067	0.136
		0.031	0.065	0.132
	Mean	0.030	0.065	0.133
% Diff	-16	-11	-5.2	
28 Sep 99	6 months	0.033	0.063	0.120
		0.032	0.061	0.119
		0.031	0.065	0.130
	Mean	0.032	0.063	0.123
% Diff	-11	-14	-12	

The results indicate that the accuracy after storage of more than 2 weeks exceeds 20%.

The results of the stability testing of QC samples with the same nominal anti-IIa activities at -80° C are shown in Table 5:

Table 5. Stability data at -80°C

date of assav	Time of storage	Nominal activities (IU anti-IIa/mL)		
		0.036	0.073	0.140
30 Mar 99	T0	0.032	0.073	0.134
		0.035	0.074	0.134
		0.040	0.081	0.148
	Mean	0.036	0.076	0.139
	% Diff	-0.93	4.1	-1.0
06 Apr 99	1 week	0.039	0.082	0.153
		0.039	0.085	0.148
		0.039	0.082	0.147
	Mean	0.039	0.083	0.149
	% Diff	8.3	14	6.7
13 Apr 99	2 weeks	0.039	0.082	0.150
		0.043	0.080	0.147
		0.036	0.080	0.144
	Mean	0.039	0.081	0.147
	% Diff	9.3	11	5.0
27 Apr 99	1 month	0.032	0.064	0.125
		0.033	0.065	0.127
		0.032	0.065	0.120
	Mean	0.032	0.065	0.124
	% Diff	-10	-11	-11
11 May 99	6 weeks	0.033	0.065	0.132
		0.034	0.070	0.136
		0.035	0.070	0.135
	Mean	0.034	0.068	0.134
	% Diff	-5.6	-6.4	-4.0
01 Jun 99	2 months	0.031	0.068	0.130
		0.028	0.068	0.127
		0.029	0.063	0.122
	Mean	0.029	0.066	0.126
	% Diff	-19	-9.1	-10
29 Jun 99	3 months	0.030	0.068	0.140
		0.034	0.072	0.143
		0.033	0.071	0.142
	Mean	0.032	0.070	0.142
	% Diff	-10	-3.7	1
28 Sep 99	6 months	0.036	0.073	0.137
		0.035	0.071	0.136
		0.035	0.068	0.131
	Mean	0.035	0.071	0.135
	% Diff	-1.9	-3.2	-3.8

The results indicate a satisfactory accuracy of the anti-IIa activity based assay of enoxaparin in samples stored at -80° C over the entire 6 months period tested.

Conclusion

Plasma samples for the determination of anti-IIa activity of enoxaparin should not be stored longer than 2 weeks at -20° C. However, storage of plasma samples can be extended to 6 months when the samples are kept at -80 °C. A safe time for exposing samples to room temperature has not been determined.

COMMENTS

A safe time of exposure of the samples to room temperature has not been determined

Study DOH 0519: Complement of Validation for the Determination of Enoxaparin Anti-IIa Activity in CTAD Human Plasma Using a New Thrombin Reagent

Study Investigator and Study Site: _____

b(6)

The bovine thrombin _____ used in the chromogenic assay determining the anti-IIa activity of enoxaparin is not any longer manufactured by the manufacturer _____ Thus, a new bovine thrombin manufactured by _____ and distributed by _____ I was tested.

b(4)

Objectives

To test the performance of the chromogenic assay measuring the anti-IIa activity of enoxaparin using the new bovine thrombin, _____

b(4)

Methods

To test the performance of the method using the new thrombin, a between-run assay was performed and accuracy and precision evaluated. Four QC samples were made at 0.020, 0.030, and 0.118 IU anti-IIa/mL. A QC sample at 0.495 IU anti-IIa activity/mL to be diluted (1:6) was also tested. The impact of freeze and thaw cycles on the performance of the assay was also evaluated. The QC samples were stored at -80° C. The assay method was as described in report DMPK/FR/2396, except for the change in thrombin, was used.

Results

The results on between-run accuracy and-precision of the assay using the new thrombin are shown in Table 3:

Table 3 – Between run assay - Quality control back calculated activity (IU anti-Ila/mL)

Date of assay	Run number	Nominal anti-Ila activity (IU anti-Ila/mL)			
		0.020	0.030	0.119	0.495
15-Nov-05	THRO1P2	0.020	0.033	0.121	0.474
	THRO1P3	0.015	0.021	0.114	0.471
	THRO1P3	0.019	0.025	0.115	0.488
16-Nov-05	THRO2P1	0.024	0.029	0.119	0.428
	THRO2P2	0.019	0.021	0.121	0.437
	THRO2P2	0.018	0.028	0.117	0.452
17-Nov-05	THRO4P1	0.023	0.031	0.129	0.517
	THRO4P2	0.022	0.027	0.134	0.565
	THRO4P2	0.031	0.035	0.141	0.557
17-Nov-05	THRO5P1	0.026	0.031	0.122	0.499
	THRO5P2	0.021	0.030	0.115	0.462
	THRO5P2	0.025	0.031	0.106	0.483
20-Dec-05	THRO6P4	0.023	0.032	0.136	0.534
	THRO6P4	0.024	0.031	0.132	0.523
	THRO6P4	0.025	0.033	0.132	0.534
21-Dec-05	THRO7P1	0.023	0.030	0.124	0.515
	THRO7P2	0.022	0.032	0.130	0.534
	THRO7P2	0.024	0.025	0.134	0.564
Mean		0.022	0.029	0.125	0.502
SD		0.004	0.004	0.009	0.042
%CV		16	14	7.5	8.3
n		18	18	18	18
Min		0.015	0.021	0.106	0.428
Max		0.031	0.035	0.141	0.564
%Diff		12	-2.8	4.7	1.3

Bold: out of acceptance criteria

The between-run accuracy ranged between -2.8% and 12% and the between-run precision was < 16%, respectively.

The results of the impact of freeze-thaw cycles on the anti-Ila activity of samples kept at -80° C are shown in Table 4:

Table 4 – Freeze / thaw cycles stability evaluation

Date of assay	Run number	Cycle of freeze/thaw	Anti-Ila activity (IU anti-Ila/mL)		
			0.030	0.119	0.495
13-Dec-05	EXT2A61	TO	0.034	0.121	0.531
			0.034	0.118	0.493
			0.031	0.125	0.541
		Mean	0.033	0.121	0.522
		SD	0.002	0.004	0.025
	%CV	5.2	2.9	4.9	
	%Diff	10	2.0	5.4	
20-Dec-05	THRO6P1 THRO6P2 THRO6P3 THRO6P4	1 cycle freeze/thaw	0.030	0.124	0.510
			0.031	0.120	0.419
			0.031	0.126	0.457
		Mean	0.031	0.123	0.462
		SD	0.001	0.003	0.046
		%CV	1.9	2.5	9.9
		%Diff	2.2	3.6	-6.7
		2 cycles freeze/thaw	0.035	0.128	0.515
			0.031	0.128	0.502
			0.030	0.127	0.548
		Mean	0.032	0.128	0.522
		SD	0.003	0.001	0.024
		%CV	8.3	0.45	4.5
		%Diff	6.7	7.3	5.4
		3 cycles freeze/thaw	0.034	0.133	0.578
			0.031	0.134	0.570
			0.033	0.134	0.567
Mean	0.033	0.134	0.572		
SD	0.002	0.001	0.006		
%CV	4.7	0.43	0.99		
%Diff	8.9	12	15		
4 cycles freeze/thaw	0.034	0.129	0.510		
	0.035	0.125	0.510		
	0.039	0.126	0.505		
Mean	0.036	0.127	0.508		
SD	0.003	0.002	0.003		
%CV	7.3	1.6	0.57		
%Diff	20	6.4	2.7		

The results indicate that the anti-IIa activity of enoxaparin in samples frozen at -80 °C and exposed to up to 4 freeze/thaw cycles is not adversely affected.

Conclusion

The performance of the anti-IIa based activity of enoxaparin using a new thrombin agent is acceptable. The anti-IIa activity levels of enoxaparin in plasma samples stored at -80°C are stable when exposed to up to 4 freeze/thaw cycles.

COMMENTS

None.

Study DMPK/FR/2396 RP54563: Chromogenic Anti-IIa Assay with Antithrombin Supplementation: Adaptation of the Assay Consecutive to a Change of the Bovine Thrombin Manufacturing Process

Study Investigator and Study Site:  

b(6)

In January 2000, the manufacturing process of the bovine thrombin was modified by the manufacturer  to increase its purity. When the new thrombin was used as described in the validation report J  the accuracy of the assay was found to be less satisfactory, although it was still within the acceptance criteria. After changing the volume of plasma from 80 µL to 70 µL and the volume of thrombin from 60 µL to 70 µL, the accuracy performance of the assay was improved.

b(4)

Objectives

To determine between-run accuracy and -precision of the assay using the modified volumes

Methods

Quality control samples of 0.020, 0.040, 0.100 and 0.500 IU anti-IIa/mL were prepared. A quality control sample with 0.500 IU anti-IIa activity was diluted (1:4 or 1:5). Results from 6 different runs were evaluated.

The results on between-run accuracy and-precision are shown in Table 5:

Table 5 - Back-calculated activities (IU anti-IIa/mL) of quality controls

Date of assay	File name	Nominal activities (IU anti-IIa/mL)			
		0.020	0.040	0.100	0.500
19-Jul-00	IHHOM1	-	0.038	0.101	0.454
24-Jul-00	IHHOM2	-	0.035	0.089	0.466
15-Sep-00	IHHOM5	0.022	0.036	0.091	0.485
18-Sep-00	IHHOM6	0.022	0.042	0.088	0.483
18-Sep-00	IHHOM7	0.020	0.037	0.095	0.486
18-Sep-00	IHHOM8	0.023	0.037	0.089	0.476
	Mean	0.022	0.038	0.092	0.475
	SD	0.001	0.002	0.005	0.013
	% C.V.	5.8	6.5	5.4	2.7
	n	4	6	6	6
	Min	0.020	0.035	0.088	0.454
	Max	0.022	0.042	0.101	0.486
	% Diff	8.7	-6.2	-7.8	-5.0

- : not assayed

The between run accuracy ranged between -7.8% and 8.7% and the between-run precision was $\leq 6.5\%$.

Conclusion

The performance of the anti-IIa activity based chromogenic assay of enoxaparin using modified volumes of plasma and thrombin is satisfactory.

Comments

None

Study DMPK/FR/2162: Validation of Anti-IIa Chromogenic Assay for the Determination of Enoxaparin RP54563 in Human Urine

Study Investigator and Study Site: ?

b(6)

Objectives

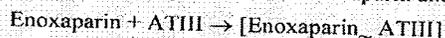
Description of a specific amidolytic anti-IIa activity assay with antithrombin III supplementation developed to measure the anti-IIa activity of enoxaparin in urine.

Two assays were developed, a first assay with an ethanol extraction step to precipitate the glycosaminoglycans and a second assay without extraction.

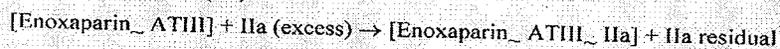
Methods

The principle of the three step procedure is shown below:

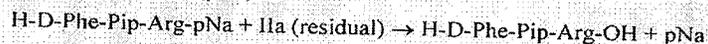
- The formation of a binary complex between enoxaparin and AT III in urine



- The formation of a ternary complex between [Enoxaparin_ATIII] and thrombin in the presence of an known excess of thrombin (IIa).



- The hydrolysis of a specific chromogenic substrate (peptide-pNa) by residual factor IIa which leads to the release of paranitroaniline (pNa)



The quantity of pNa liberated, measured spectrophotometrically at 405 nm is inversely proportional to the concentration of enoxaparin. A linear dose-reponse curve between 0.000 and 0.200 IU anti-IIa/ml activity is obtained by plotting the nominal activities of the standards versus the absorbance logarithm.

The methodology set up combines or not a pretreatment of urine samples with alcohol precipitation according to the technique of Nishiyama *et al.* [3] before the measurement of the anti-factor IIa activity by anidolytic method.

Enoxaparin standard calibration curves in urine, constructed for each assay system, consisted of a blank urine of each subject (in order to discriminate between endogenous and exogenous components) to which the corporate standard (Batch PRS 122, lot CB 05667 and WSD 3015) was added by increasing amounts.

The assay uses human antithrombin III and S 2238 as chromogenic substrate.

For each human volunteer providing urine with anti-IIa activity calibration curves were set up using their blank urine. QC samples were made up from human urine containing nominal anti-IIa activities of 0.020 0.050, 0.100 and 0.200 IU/mL (Lot CB 05667) or 0.024, 0.048, 0.096, 0.144 and 0.192 IU/mL (Lot WSD 3015). The QC samples were immediately frozen and kept at -80 ° C and then exposed to 1 or 2 freeze/thaw cycles.

The extraction procedure consisted in mixing 2 mL of urine with 5 mL of absolute alcohol. After centrifugation at 1200 g for 10 min the precipitate was dissolved with 2 mL NaCl isotonic solution and then ultrasonicated until complete dissolution.

A linear least square regression was used to establish the linearity of the calibration curve.

The within-day-and between day- accuracy and precision of the method were evaluated by measuring the anti-IIa activity of the QC samples.

The LLOQ was defined as the lowest anti-IIa activity which can be measured with an accuracy within 20% of the nominal activity and a precision of $\leq 20\%$.

The extraction yield was determined by reading the urine standards with respect to a calibration curve in NaCl isotonic solution. The yield of recovery was calculated in relation to the nominal values. The effect of keeping the plasma samples for 3 h at room temperature prior to analysis and the impact of two freeze/thaw cycles on the stability of the samples kept at $-80\text{ }^{\circ}\text{C}$ were also tested.

Results

Procedure without Extraction

There was an inverse linear relationship between anti-IIa activity and $\Delta A_{405\text{ nm}}$.

Based on the results on accuracy and precision of the calibration standards shown in Tables 3 and 8 the LLOQ was set at 0.040 IU anti-IIa/mL:

Table 3 :

**Within-day assay reproducibility of calibration curve without extraction step
Back calculated activities of each $\Delta A_{405\text{ nm}}$ related to the mean calibration curve
(November 16, 1994)**

File name	Nominal activity (IU anti-IIa/ml)							
	0.010	0.020	0.040	0.060	0.080	0.100	0.150	0.200
UIIa16/A,B	0.016	0.022	0.037	0.057	0.077	0.088	0.151	0.192
UIIa16/C,D	0.017	0.017	0.034	0.048	0.072	0.090	0.148	0.187
UIIa16/E,F	0.017	0.024	0.036	0.057	0.072	0.091	0.151	0.195
UIIa16/G,H	0.013	0.019	0.037	0.057	0.071	0.091	0.158	0.200
UIIa16/I,J	0.014	0.022	0.043	0.056	0.082	0.094	0.161	0.203
UIIa16/K,L	0.010	0.024	0.039	0.057	0.088	0.096	0.160	0.209
UIIa16/M,N	0.011	0.019	0.036	0.054	0.072	0.095	0.164	0.213
UIIa16/O,P	0.008	0.018	0.038	0.059	0.076	0.090	0.157	0.209
UIIa16/Q,R	0.019	0.022	0.038	0.058	0.080	0.098	0.149	0.211
Mean	0.014	0.021	0.038	0.056	0.077	0.093	0.155	0.202
SD	0.004	0.003	0.003	0.003	0.006	0.003	0.006	0.009
%CV	27	12	7	6	8	4	4	5
n	9	9	9	9	9	9	9	9
Min	0.008	0.017	0.034	0.048	0.071	0.088	0.148	0.187
Max	0.019	0.024	0.043	0.059	0.088	0.098	0.164	0.213
% relative error	39	4	-6	-7	-4	-7	4	1

Table 8 :

**Between-day assay reproducibility of calibration curve without extraction step
Back calculated activities of each Δ A 405nm related to each calibration curve**

Date of assays	File name	Nominal activity (IU anti-IIa/ml)							
		0.010	0.020	0.040	0.060	0.080	0.100	0.150	0.200
18 Oct 94	UIIa18/A,B	0.011	0.025	0.041	0.055	0.077	0.094	0.142	0.210
18 Oct 94	UIIa18/E,F,G	0.012	0.016	0.033	0.063	0.073	0.097	0.158	0.198
18 Oct 94	UIIa18/I,J	0.012	0.022	0.041	0.050	0.077	0.093	0.156	0.201
21 Oct 94	UIIa21/A,B,C	0.013	0.018	0.038	0.058	0.078	0.098	0.159	0.195
21 Oct 94	UIIa21/E,F	0.012	0.023	0.036	0.057	0.076	0.101	0.152	0.200
21 Oct 94	UIIa21/I,J,K	0.012	0.025	0.041	0.051	0.083	0.093	0.150	0.203
	mean	0.012	0.022	0.038	0.056	0.077	0.096	0.153	0.201
	S.D.	0.001	0.004	0.003	0.005	0.003	0.003	0.006	0.005
	%CV	5	17	9	9	4	3	4	3
	n	6	6	6	6	6	6	6	6
	Min	0.011	0.016	0.033	0.050	0.073	0.093	0.142	0.195
	Max	0.013	0.025	0.041	0.063	0.083	0.101	0.159	0.210
	%relative error	20	8	-4	-7	-3	-4	2	1

However, the 0.010 and 0.020 IU anti-II/mL levels were used in the linear regression of the calibration curve.

As shown in Tables 5 and 10 the within-day and between-day accuracy of the QC samples with 0.050-0.200 IU anti-II activity/mL ranged between -7% and 3% and 8% and 14 %, respectively. The corresponding precision values were $\leq 8\%$ and $\leq 17\%$, respectively:

Table 5 :

**Within-day assay reproducibility of quality controls without extraction step
Back calculated activities of each Δ A 405nm related to each calibration curve
(November 16, 1994)**

File name	Nominal activity (IU anti-IIa/ml)			
	0.020	0.050	0.100	0.200
UIIa16/A,B	0.015	0.043	0.102	0.205
UIIa16/C,D	0.018	0.046	0.102	0.211
UIIa16/E,F	0.024	0.053	0.103	0.211
UIIa16/G,H	0.021	0.052	0.103	0.203
UIIa16/I,J	0.019	0.047	0.097	0.211
UIIa16/K,L	0.020	0.044	0.100	0.205
UIIa16/M,N	0.020	0.046	0.097	0.197
UIIa16/O,P	0.016	0.044	0.100	0.196
UIIa16/Q,R	0.014	0.043	0.093	0.214
Mean	0.019	0.046	0.100	0.206
SD	0.003	0.004	0.003	0.006
%CV	17	8	3	3
n	9	9	9	9
Min.	0.014	0.043	0.093	0.196
Max.	0.024	0.053	0.103	0.214
% relative error	-7	-7	0	3

Table 10 :

**Between-day assay reproducibility of quality controls without extraction step
Back calculated activities of each Δ A 405nm related to each calibration curve**

Date of assays	File name	Nominal activity (IU anti-IIa/ml)			
		QC 0.020	QC 0.050	QC 0.100	QC 0.200
18 Oct 94	UIIa18/A,B	0.029	0.064	0.119	0.223
18 Oct 94	UIIa18/E,F,G	0.026	0.045	0.100	0.188
18 Oct 94	UIIa18/I,J	0.022	0.045	0.117	0.220
21 Oct 94	UIIa21/A,B,C	0.017	0.048	0.113	0.237
21 Oct 94	UIIa21/E,F	0.028	0.064	0.127	0.259
21 Oct 94	UIIa21/I,J,K	0.025	0.059	0.108	0.204
	mean	0.025	0.054	0.114	0.222
	S.D	0.004	0.009	0.009	0.025
	%CV	18	17	8	11
	n	6	6	6	6
	Min	0.017	0.045	0.100	0.188
	Max	0.029	0.064	0.127	0.259
	% relative error	23	8	14	11

To check the possible impact of endogenous glycosaminoglycans on the performance of the chromogenic assay the apparent anti-IIa activity of blank urine was determined. A value of < 0.010 IU anti-IIa activity/mL was estimated and considered negligible.

The specificity of the assay depends on the specificity of the oligopeptide used as substrate for factor IIa thrombin. The report states that the oligomer S 2238 is specific for thrombin, but does not provide data supporting the statement.

Procedure with Extraction Step

There was an inverse linear relationship between anti-IIa activity and ΔA_{405} nm.

Based on the results on accuracy and precision of the calibration standards shown in Tables 13 and 18 the LLOQ was set at 0.048 or 0.51 IU anti-IIa/mL dependent on the batch of enoxaparin used:

Table 13 :

**Within-day assay reproducibility of calibration curve with extraction step
Back calculated activities of each Δ A 405nm related to each calibration curve
(August 18, 1997)
Lot WSD 3015**

File name	Nominal activity (IU anti-IIa/ml)				
	0.024	0.048	0.096	0.144	0.192
IlaUIA1/A,B	0.021	0.041	0.116	0.134	0.188
IlaUIA2/A,B	0.021	0.044	0.096	0.144	0.193
IlaUIA3/A,B	0.024	0.050	0.095	0.137	0.197
IlaUIA4/A,B	0.024	0.043	0.097	0.141	0.195
IlaUIA5/A,B	0.018	0.048	0.098	0.154	0.184
IlaUIA6/A,B	0.027	0.042	0.088	0.156	0.187
Mean	0.023	0.045	0.098	0.144	0.191
SD	0.003	0.004	0.009	0.009	0.005
%CV	14	8	10	6	3
n	6	6	6	6	6
Min.	0.018	0.041	0.088	0.134	0.184
Max.	0.027	0.050	0.116	0.156	0.197
% relative error	-6	-7	2	0	-1

Table 18 :

**Between-day assay reproducibility of calibration curve with extraction step
Back calculated activities of each Δ A 405nm related to each calibration curve
Lot CB 05667**

Date of assays	File name	Nominal activity (IU anti-IIa/ml)				
		0.025	0.051	0.101	0.152	0.203
27 Mar 95	AEUIIa1/C.D	0.022	0.047	0.105	0.157	0.199
29 Mar 95	AEUIIa2/C.D	0.026	0.043	0.099	0.155	0.203
29 Mar 95	AEUIIa3/C.D	0.022	0.044	0.103	0.162	0.195
29 Mar 95	AEUIIa4/C.D	0.030	0.038	0.094	0.164	0.198
31 Mar 95	AEUIIa5/C.D	0.035	0.039	0.086	0.155	0.207
31 Mar 95	AEUIIa6/C.D	0.019	0.043	0.104	0.157	0.199
	mean	0.026	0.042	0.099	0.158	0.200
	S.D.	0.006	0.003	0.007	0.004	0.004
	%CV	23	8	7	2	2
	n	6	6	6	6	6
	Min	0.019	0.038	0.086	0.155	0.195
	Max	0.035	0.047	0.105	0.164	0.207
	%relative error	3	-17	-2	4	-1

The results on the within-day and between-day accuracy and -precision of the QC samples of Lots WSD 3015 and CB 05667 are shown in Tables 15 ad 20:

Table 15 :

**Within-day assay reproducibility of quality controls with extraction step
Back calculated activities of each Δ A 405nm related to each calibration curve
(August 18, 1997)
Lot WSD 3015**

File name	Nominal activity (IU anti-IIa/ml)				
	0.024	0.048	0.096	0.144	0.192
IaUIA1/A.B	0.020	0.031	0.078	0.125	0.163
IaUIA2/A.B	0.019	0.037	0.082	0.134	0.165
IaUIA3/A.B	0.025	0.031	0.073	0.116	0.203
IaUIA4/A.B	0.022	0.035	0.113	0.128	0.167
IaUIA5/A.B	0.030	0.040	0.095	0.133	0.174
IaUIA6/A.B	0.028	0.039	0.091	0.115	0.173
Mean	0.024	0.036	0.089	0.125	0.174
SD	0.004	0.004	0.014	0.008	0.015
%CV	18	11	16	7	8
n	6	6	6	6	6
Min	0.019	0.031	0.073	0.115	0.163
Max	0.030	0.040	0.113	0.134	0.203
% relative error	0	-26	-8	-13	-9

Table 20 :

**Between-day assay reproducibility of quality controls with extraction step
Back calculated activities of each Δ A 405nm related to each calibration curve
Lot CB 05667**

Date of assays	File name	Nominal activity (IU anti-IIa/ml)	
		QC 0.051	QC 0.203
27 Mar 95	AEUIIa1/C,D	0.051	0.176
29 Mar 95	AEUIIa2/C,D	0.043	0.203
29 Mar 95	AEUIIa3/C,D	0.044	0.212
29 Mar 95	AEUIIa4/C,D	0.048	0.234
31 Mar 95	AEUIIa5/C,D	0.057	0.298
31 Mar 95	AEUIIa6/C,D	0.041	0.215
	mean	0.047	0.223
	S.D.	0.006	0.041
	%CV	13	19
	n	6	6
	Min	0.041	0.176
	Max	0.057	0.298
	% relative error	-7	10

The results indicated that the between-day accuracy at the 0.096, 0.144 and 0.192 IU anti-IIa activity levels (Lot WSD 3015) range between -13% and -8%. The between-day accuracy at the 0.051 and 0.203 anti-IIa activity/mL levels (Lot CB 05667) ranged between -7% and 10%. The corresponding values for precision were $\leq 16\%$ and $\leq 19\%$. The estimated extraction yield ranged between 80.6% and 112.5%.

The impact of exposing QC urine samples (Lot WSD 3015, assay with extraction step) to room temperature for 3 h is shown in Table 21:

Table 21 :

Robustness assay on computed activity of quality controls with extraction step

a) Assays performed extemporaneously (t=0)

File name	nominal activity (IU anti-IIa/ml)				
	0.024	0.048	0.096	0.144	0.192
IURUB1/A,B	0.022	0.043	0.096	0.155	0.184
IURUB1/A,B	0.019	0.032	0.085	0.142	0.169
IURUB2/A,B	0.023	0.044	0.090	0.145	0.195
IURUB2/A,B	0.024	0.048	0.084	0.141	0.178
IURUB3/A,B	0.022	0.042	0.096	0.140	0.196
IURUB3/A,B	0.021	0.042	0.078	0.127	0.165
mean	0.022	0.042	0.088	0.142	0.181
SD	0.002	0.005	0.007	0.009	0.013
CV%	8	13	8	6	6
n	6	6	6	6	7
Min	0.019	0.032	0.078	0.127	0.165
Max	0.024	0.048	0.096	0.155	0.196
% relative error	-9.0	-12.8	-8.2	-1.6	-5.6

b) Assays performed after 3 hours at the laboratory temperature (30°C)

File name	nominal activity (IU anti-IIa/ml)				
	0.024	0.048	0.096	0.144	0.192
IURUB4/A,B	0.014	0.031	0.085	0.130	0.184
IURUB4/A,B	0.014	0.027	0.079	0.126	0.172
IURUB5/A,B	0.019	0.040	0.092	0.138	0.173
IURUB5/A,B	0.017	0.036	0.081	0.130	0.164
IURUB6/A,B	0.018	0.038	0.095	0.139	0.193
IURUB6/A,B	0.021	0.035	0.087	0.130	0.178
mean	0.017	0.035	0.087	0.132	0.177
SD	0.003	0.005	0.006	0.005	0.010
CV%	16	14	7	4	6
n	6	6	6	6	6
Min	0.014	0.027	0.079	0.126	0.164
Max	0.021	0.040	0.095	0.139	0.193
% relative error	-28.5	-28.1	-9.9	-8.2	-7.6

The results showed acceptable accuracy and precision only at the 0.048 -0.192IU anti-II activity/mL levels indicating stability of urine samples exposed to room temperature for a 3 h time period.

The impact of freeze/thaw cycles on the stability of urine samples (Lot WSD 3015, assay with extraction step) kept at -80° C is shown in Table 22:

Table 22 :
Stability assay performed on cycle of freezing-thawing with extraction step

a) Computed activity of fresh quality controls with extraction step

File name	nominal activity (IU anti-IIa/ml)				
	0.024	0.048	0.096	0.144	0.192
IURUB1/A.B	0.019	0.032	0.085	0.142	0.169
IURUB2/A.B	0.024	0.048	0.084	0.141	0.178
IURUB3/A.B	0.021	0.042	0.078	0.127	0.165
mean	0.021	0.041	0.082	0.137	0.171
SD	0.003	0.008	0.004	0.008	0.007
CV%	12	20	5	6	4
n	3	3	3	3	3
Min	0.019	0.032	0.078	0.127	0.165
Max	0.024	0.048	0.085	0.142	0.178
% relative error	-11.1	-15.3	-14.2	-5.1	-11.1

b) Computed activity of quality controls with extraction step after one cycle

File name	nominal activity (IU anti-IIa/ml)				
	0.024	0.048	0.096	0.144	0.192
IURUB4/A.B	0.009	0.027	0.070	0.109	0.151
IURUB5/A.B	0.010	0.029	0.071	0.117	0.174
IURUB6/A.B	0.012	0.028	0.079	0.138	0.169
mean	0.010	0.028	0.073	0.121	0.165
SD	0.002	0.001	0.005	0.015	0.012
CV%	15	4	7	12	7
n	3	3	3	3	3
Min	0.009	0.027	0.070	0.109	0.151
Max	0.012	0.029	0.079	0.138	0.174
% relative error	-56.9	-41.7	-23.6	-15.7	-14.2

b) Computed activity of quality controls with extraction step after two cycles

File name	nominal activity (IU anti-IIa/ml)				
	0.024	0.048	0.096	0.144	0.192
IURUB7/A.B	0.018	0.038	0.082	0.132	0.164
IURUB8/A.B	0.023	0.038	0.091	0.134	0.164
IURUB9/A.B	0.017	0.035	0.072	0.117	0.142
mean	0.019	0.037	0.082	0.128	0.157
SD	0.003	0.002	0.010	0.009	0.013
CV%	17	5	12	7	8
n	3	3	3	3	3
Min	0.017	0.035	0.072	0.117	0.142
Max	0.023	0.038	0.091	0.134	0.164
% relative error	-19.4	-22.9	-14.9	-11.3	-18.4

The results show an impact of one or two freeze/thaw cycles on the accuracy of the chromogenic assay method indicating that the anti-II activity of enoxaparin is not stable when exposed to one or more freeze/thaw cycles.

Conclusion

Accuracy and precision of the assay with the extraction step in urine samples kept at room temperature for up to 3 h are acceptable. However, the stability of the anti-IIa

activity of enoxaparin in urine samples kept at -80° C and exposed to one or more freeze/thaw cycles is compromised. Only the procedure without the extraction step provided acceptable accuracy and precision, however its performance in samples exposed to room temperature and freeze/thaw cycles was not tested.

Study: _____ Design and Evaluation of Analytical Methods Dedicated to the Pharmacokinetics of Enoxaparin in Man, Clotting Methods, Amidolytic Methods (Anti-Xa and Anti-IIa or Antithrombin Activity)

b(4)

Study Investigator and Study Site: _____

b(6)

This report characterizes the chromogenic assays measuring the anti-Xa and anti-IIa activities of enoxaparin in plasma and the impact of enoxaparin on aPTT. Because the above reviewed reports covered chromogenic assays in plasma the review of the present report focussed on the assay of aPTT.

Objectives

To describe the aPTT test

aPTT is a general coagulation screening test of the intrinsic pathway including different serine proteases such as factors XII, XI, IX, VIII, X, V, II and fibrinogen. The principle of the method involves recalcification of plasma in the presence of standardized amounts of platelet F3 substitute and specific factor XII activator either particulate or soluble. After incubation of platelet substitute and plasma for a specified time period, the reaction is initiated by addition of calcium ions. The time required for clot formation is measured. Clot formation is monitored by recording the scattering of light during the process of clot formation.

The reagents used include thromboplastin, calcium chloride (0.25 M), calibration plasma and quality control "normal" and "abnormal" citrated plasma. Test and QC samples are assayed simultaneously. The results are expressed in seconds.

The impact of the anticoagulant, citrate or citrate-theophylline-adenosine-dipyridamol on clotting time was measured as shown in Table 1:

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ANTICOAGULANTS	CLOTTING TIME mean \pm σ (s)	C.V. %
citrate solution (n = 80)	36.44 \pm 4.30 r = 29 - 48	11.8
CTAD mixture (Diatube R) (n = 32)	28.75 \pm 2.10 r = 25 - 33	7.3

σ : Standard deviation

C.V. : Coefficient of variation

r : range

TABLE 1 : Activated Partial Thromboplastin Time (A.P.T.T.) :
A.P.T.T. clotting time normal values measured on "ex-vivo" samples from healthy volunteers meeting the inclusion criteria for enoxaparin biological activity studies. Influence of the anticoagulant used for plasma collection.

Normal range plasma values were defined as 95% confidence interval range with ± 2 SD from the mean aPTT value obtained by assaying "blank" plasma collected from healthy volunteers. The reference normal ranges are respectively 32-41 sec, when citrated blood is collected and 26-31 sec, when CTAD blood is collected.

Three plasma with normal or prolonged aPTT characteristics were evaluated 7 times in the same run (within-assay reproducibility) or on 23 repeated assays (between-assay reproducibility). The control samples consisted of commercially available _____ as recommended for monitoring coagulation tests. The results on within-assay- and between-assay reproducibility are shown in Table 2:

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PLASMA SAMPLE	WITHIN ASSAY (n = 7) Clotting time (s)		BETWEEN ASSAY (n = 23) Clotting time (s)	
	mean ± σ	C.V.%	mean ± σ	C.V.%
Plasma pool	29.94 ± 0.20	0.7	30.40 ± 0.74	2.4
I.L.	r = 29.7 - 30.4		r = 29.5 - 32.0	
	30.36 ± 0.18	0.6	31.10 ± 0.89	2.9
	r = 30.2 - 30.7		r = 29.8 - 32.8	
	66.58 ± 0.85	1.3	66.79 ± 2.10	3.1
	r = 65.4 - 67.7		r = 62.2 - 70.2	

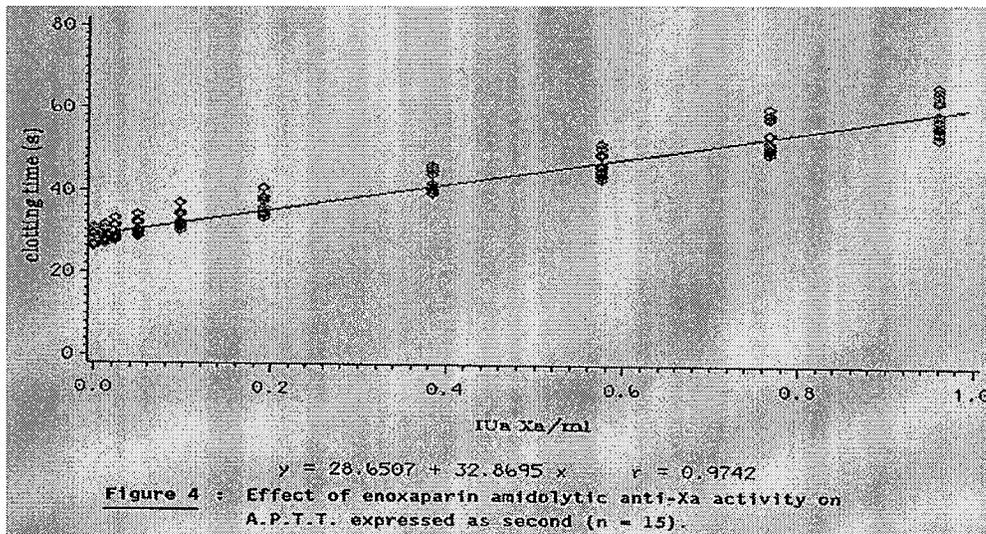
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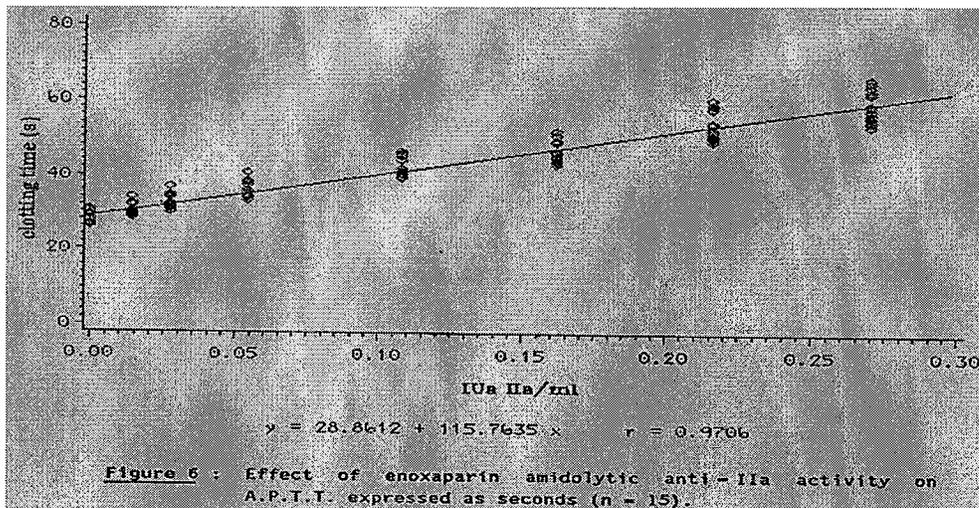
σ : Standard deviation
 C.V. % : Coefficient of variation (%)
 r : range

TABLE 2 : A.P.T.T. clotting time (seconds) :
 within-and between-assay reproducibility.

The intra-assay and inter-assay reproducibility was ≤ 1.3% and 2.9%, respectively, with normal plasma. The respective values for abnormal plasma were 1.3% and 3.1%. The precision values were within the 5% limit defined for normal plasma at the 2nd International Symposium on Standardization and Quality Control of Coagulation Tests in Rome (1989). In the presence of enoxaparin the precision of the aPTT was increased to 6%.

Statistically significant correlations existed between the effect of enoxaparin on aPTT and the anti-IIa and anti-IXa activities of enoxaparin measured by the chromogenic assays as shown in Figures 4 and 6:





Conclusion

The precision values for aPTT in the absence of enoxaparin met the standards for normal plasma presented at the 2nd International Symposium on Standardization and Quality Control of Coagulation Tests in Rome, 1989. Mean precision of aPTT in the presence of enoxaparin was 6%. The sponsor did not evaluate the performance of their aPTT test relative to today's standards. Factors affecting the sample stability were examined. In conclusion the reported values on aPTT must be interpreted with caution.

Comments

None

Upon Request from the Reviewer the Sponsor provided the following additional Information regarding the anti-Xa and IIa Activity based Enoxaparin Assays:

1. Contribution of endogenous mucopolysaccharides to the anti-Xa and anti-IIa activity based assays for enoxaparin

The below 2 Tables from study report RP54563Q-142 compare the background values from endogenous mucopolysaccharides measured in baseline samples to the respective LLOQ values of the anti-Xa and anti-IIa activities. The results indicate that the contribution of endogenous mucopolysaccharides to the anti-Xa activity based assay is negligible whereas the background by endogenous mucopolysaccharides in baseline samples relative to the LLOQ of the anti-IIa activity assay is about 40%.

Chromogenic anti-Xa assay – Data from study RP54563Q-142

Date of assay	Subject number	Subject baseline		Standard 0	Standard 0.025 IU anti-Xa/mL (LLOQ)	
		Absorbance	Anti-Xa back calculated activity (IU anti-Xa/mL)	Absorbance	Absorbance	Anti-Xa back calculated activity (IU anti-Xa/mL)
26-Jan-99	00001					
27-Jan-99	00002					
01-Feb-99	00003					
02-Feb-99	00004					
03-Feb-99	00005					
03-Feb-99	00006					
04-Feb-99	00007					
04-Feb-99	00008					
05-Feb-99	00009					
08-Feb-99	00010					
09-Feb-99	00011					
09-Feb-99	00012					
10-Feb-99	00013					
10-Feb-99	00014					
11-Feb-99	00015					
12-Feb-99	00016					

b(4)

Chromogenic anti-IIa assay – Data from study RP54563Q-142

Date of assay	Subject number	Subject baseline		Standard 0	Standard 0.020 IU anti-IIa/mL (LLOQ)	
		Absorbance	Anti-IIa back calculated activity (IU anti-IIa/mL)	Absorbance	Absorbance	Anti-IIa back calculated activity (IU anti-IIa/mL)
26-Jan-99	00001					
27-Jan-99	00002					
01-Feb-99	00003					
02-Feb-99	00004					
03-Feb-99	00005					
03-Feb-99	00006					
04-Feb-99	00007					
04-Feb-99	00008					
05-Feb-99	00009					
08-Feb-99	00010					
09-Feb-99	00011					
09-Feb-99	00012					
10-Feb-99	00013					
10-Feb-99	00014					
11-Feb-99	00015					
12-Feb-99	00016					

b(4)

2. Conditions that guarantee stability of plasma samples before, during and after analysis

The below two Tables indicate that the anti-Xa and anti-IIa activities in plasma samples kept at 4 ° C during analysis runs of 90 min can be considered stable:

Table 1: Anti-Xa chromogenic assay in human plasma – RP54563Q-142 study – Quality Controls

Anti-IIa with ATIII chromogenic assay in human plasma - Study 142
 Stability at +4°C throughout the analytical run evaluated on human plasma quality controls
 Duration of the analytical run: maximum of 1h30 - 1h as a mean

Date of assay	File name	First set of QC			Last set of QC			%Diff (Last QC-First QC)/First QC		
		0.040	0.100	0.500	0.040	0.100	0.500	0.040	0.100	0.500
26-jan-99	IVIIa1 / IV21P4	0.033	0.107	0.507	0.039	0.114	0.575	18	6.5	13
27-jan-99	IVIIa2 / IV22P4	0.033	0.099	0.527	0.034	0.107	0.501	3.0	8.1	-4.9
01-feb-99	IVIIa3 / IV23P4	0.037	0.100	0.519	0.034	0.095	0.485	-8.1	-5.0	-6.6
02-feb-99	IVIIa4 / IV24P4	0.033	0.104	0.516	0.037	0.113	0.557	12	8.7	7.9
03-feb-99	IVIIa5 / IV25P4	0.037	0.103	0.505	0.044	0.110	0.502	19	6.8	-0.59
03-feb-99	IVIIa6 / IV26P4	0.038	0.095	0.470	0.042	0.110	0.558	11	16	19
04-feb-99	IVIIa7 / IV27P4	0.030	0.107	0.528	0.037	0.104	0.551	NA	-2.8	4.4
04-feb-99	IVIIa8 / IV28P4	0.028	0.080	0.431	0.037	0.091	0.501	NA	14	16
05-feb-99	IVIIa9 / IV29P4	0.027	0.098	0.494	0.034	0.100	0.481	NA	2.0	-2.6
08-feb-99	IVIIa10 / IV210P4	0.037	0.112	0.564	0.039	0.121	0.593	5.4	8.0	5.1
09-feb-99	IVIIa11 / IV211P4	0.038	0.105	0.484	0.037	0.118	0.534	-2.6	12	10
09-feb-99	IVIIa12 / IV212P4	0.041	0.111	0.560	0.039	0.116	0.571	-4.9	4.5	2.0
10-feb-99	IVIIa13 / IV213P4	0.039	0.099	0.504	0.039	0.115	0.558	0.0	16	11
10-feb-99	IVIIa14 / IV214P4	0.035	0.105	0.506	0.033	0.104	0.511	-5.7	-1.0	1.0
11-feb-99	IVIIa15 / IV215P4	0.034	0.104	0.536	0.030	0.099	0.514	-12	-4.8	-4.1
12-feb-99	IVIIa16 / IV216P4	0.040	0.110	0.542	0.038	0.109	0.514	-5.0	-0.91	-5.2
Mean								2.3	5.5	4.1

Bold : out of acceptance criteria
 NA : not applicable

Table 2: Anti-IIa chromogenic assay in human plasma – RP54563Q-142 study – Quality Controls

Anti-IIa with ATIII chromogenic assay in human plasma - Study 142
 Stability at +4°C throughout the analytical run evaluated on human plasma quality controls
 Duration of the analytical run: maximum of 1h30 - 1h as a mean

Date of assay	File name	First set of QC			Last set of QC			%Diff (Last QC-First QC)/First QC		
		0.040	0.100	0.500	0.040	0.100	0.500	0.040	0.100	0.500
26-jan-99	IVIIa1 / IV21P4	0.033	0.107	0.507	0.039	0.114	0.575	18	6.5	13
27-jan-99	IVIIa2 / IV22P4	0.033	0.099	0.527	0.034	0.107	0.501	3.0	8.1	-4.9
01-feb-99	IVIIa3 / IV23P4	0.037	0.100	0.519	0.034	0.095	0.485	-8.1	-5.0	-6.6
02-feb-99	IVIIa4 / IV24P4	0.033	0.104	0.516	0.037	0.113	0.557	12	8.7	7.9
03-feb-99	IVIIa5 / IV25P4	0.037	0.103	0.505	0.044	0.110	0.502	19	6.8	-0.59
03-feb-99	IVIIa6 / IV26P4	0.038	0.095	0.470	0.042	0.110	0.558	11	16	19
04-feb-99	IVIIa7 / IV27P4	0.030	0.107	0.528	0.037	0.104	0.551	NA	-2.8	4.4
04-feb-99	IVIIa8 / IV28P4	0.028	0.080	0.431	0.037	0.091	0.501	NA	14	16
05-feb-99	IVIIa9 / IV29P4	0.027	0.098	0.494	0.034	0.100	0.481	NA	2.0	-2.6
08-fev-99	IVIIa10 / IV210P4	0.037	0.112	0.564	0.039	0.121	0.593	5.4	8.0	5.1
09-feb-99	IVIIa11 / IV211P4	0.038	0.105	0.484	0.037	0.118	0.534	-2.6	12	10
09-feb-99	IVIIa12 / IV212P4	0.041	0.111	0.560	0.039	0.116	0.571	-4.9	4.5	2.0
10-feb-99	IVIIa13 / IV213P4	0.039	0.099	0.504	0.039	0.115	0.558	0.0	16	11
10-feb-99	IVIIa14 / IV214P4	0.035	0.105	0.506	0.033	0.104	0.511	-5.7	-1.0	1.0
11-feb-99	IVIIa15 / IV215P4	0.034	0.104	0.536	0.030	0.099	0.514	-12	-4.8	-4.1
12-feb-99	IVIIa16 / IV216P4	0.040	0.110	0.542	0.038	0.109	0.514	-5.0	-0.91	-5.2
Mean								2.3	5.5	4.1

Bold : out of acceptance criteria
 NA : not applicable

6. Pharmacometrics Review

PHARMACOMETRIC REVIEW

NDA:	22138
Drug name:	Lovenox (Enoxaparin)
Indication:	Treatment of acute ST-segment myocardial infarction (STEMI)
Proposed Regimen (Sponsor):	iv. bolus 30mg (Induction) sc 1mg/kg q 12 hr (Maintenance)
Applicant:	Sanofi Aventis
OCP Reviewer	Peter Hinderling, M.D.
PM Reviewer:	Hao Zhu, Ph.D.
PM Team Leader:	Joga Gobburu, Ph.D.
Type of Submission:	NDA
Submission Date:	Nov 17, 2006
PDUFA Date:	May 17, 2007

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1. Executive Summary

Lovenox (Enoxaparin Sodium) is a low molecular weight heparin (LMWH) obtained by depolymerization of standard heparin. It is comprised of multiple moieties with anti-factor Xa (inhibition of thrombin generation) and anti-factor IIa (inhibition of thrombin) activities. Enoxaparin has been approved for prophylaxis of deep vein thrombosis, treatment of acute deep vein thrombosis, and prophylaxis of ischemic complications of unstable angina and non-Q wave myocardial infarction.

This submission is a supplement New Drug Application (sNDA) to obtain marketing approval for the additional indication of acute ST-Segment Elevation Myocardial Infarction (STEMI).

Totally 6 population PK and PK/PD reports are included in this submission package. Studies RP54563Q-312 AMI SK, XRP4563B-267 ENTIRE-TIMI-23, XRP4563B/3001 BAY001, POH0137, and DMPK/FR/2249 TIMI 11A are population PK studies. Population exposure-efficacy relationship is explored in RP54563Q-312 AMI SK study, whereas population exposure-safety relationship is explored in both RP54563Q-312 AMI SK study and DMPK/FR/2409 TIMI 11A study.

The main safety concern for enoxaparin is major hemorrhage, including a clinically overt hemorrhage resulting in a fall of ≥ 3 gm/dL in hemoglobin, or a retroperitoneal, intracranial, or intraocular hemorrhage.

The exposure-hemorrhage analysis identified that patients with higher exposure have a higher probability of major hemorrhage. Also, patients with compromised renal function inherently have a higher risk in addition to enoxaparin exposure.

Patient risk simulation indicates that STEMI patients with severe renal impairment should be administered a normal loading dose of 30mg iv, but the sc maintenance dose should be changed to 1mg/kg q24 hr in stead of 1mg/kg q 12hr. These findings support the sponsor's proposal for dosing adjustment in the severe renal impaired patients.

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2. Question Based Review

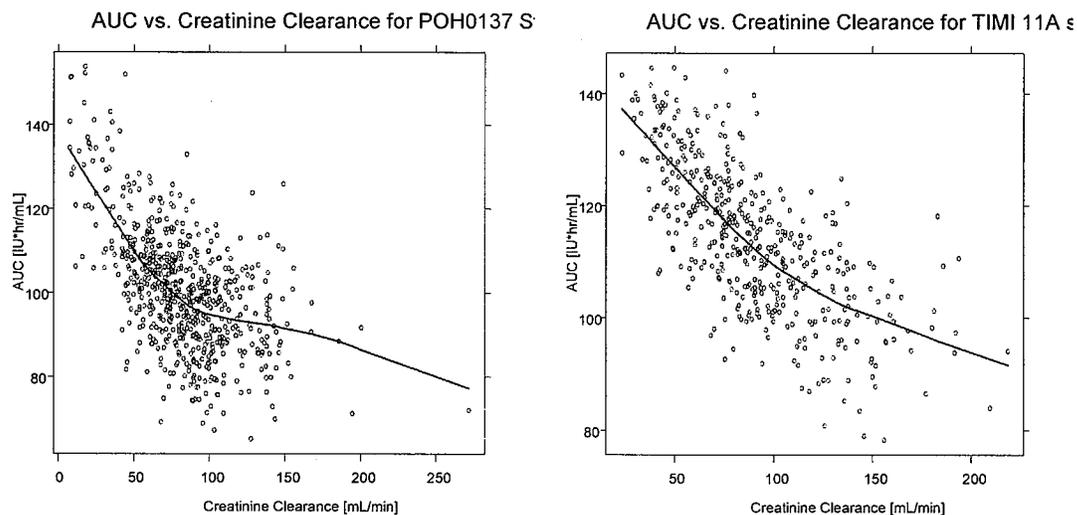
Is sponsor's proposed dosing adjustment (1mg/kg q 24 hr instead of q 12hr) reasonable for severe renal impaired patients?

The sponsor proposed using 1mg/kg q 24hr instead of q 12hr for patients with severe renal impairment. Based on the patient risk analysis, this dosing adjustment is reasonable.

For enoxaparin, although only 11% of a dose seems to be excreted by kidney in the form of active fragments, the overall impact of renal function on exposure and response appears more important.

Enoxaparin population PK analysis from POH0137 and TIMI 11A study demonstrated that the most important covariate effect on enoxaparin clearance is creatinine clearance (CrCl). In the TIMI 11A study, patients with CrCl of 50 mL/min (the threshold for moderate renal impairment) or 30 mL/min (the threshold for severe renal impairment) would have enoxaparin clearances decreased by 17% and 27%, respectively. In the POH0137 study, the most important covariate effect on enoxaparin clearance is also CrCl, with a decrease of clearance of 11% and 17% in moderate and severe renally impaired patients, respectively, resulting in an increase of 12.3 and 21.5% in AUC respectively. The influence of creatinine clearance on enoxaparin exposure (steady state AUC 0-12hr) can be illustrated in Fig 1.

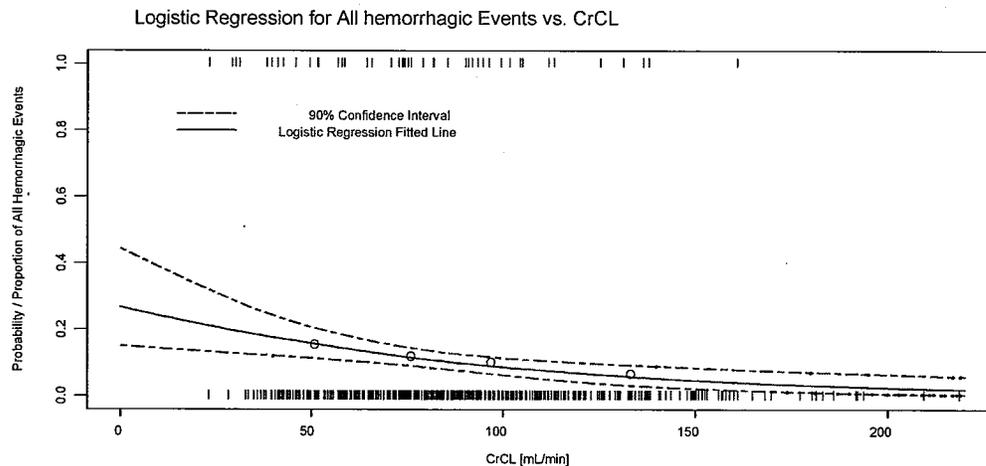
Fig 1. Effect of renal impairment on enoxaparin exposure based on population PK study POH0137 and TIMI 11A



Note: the read line is the lowess smooth line

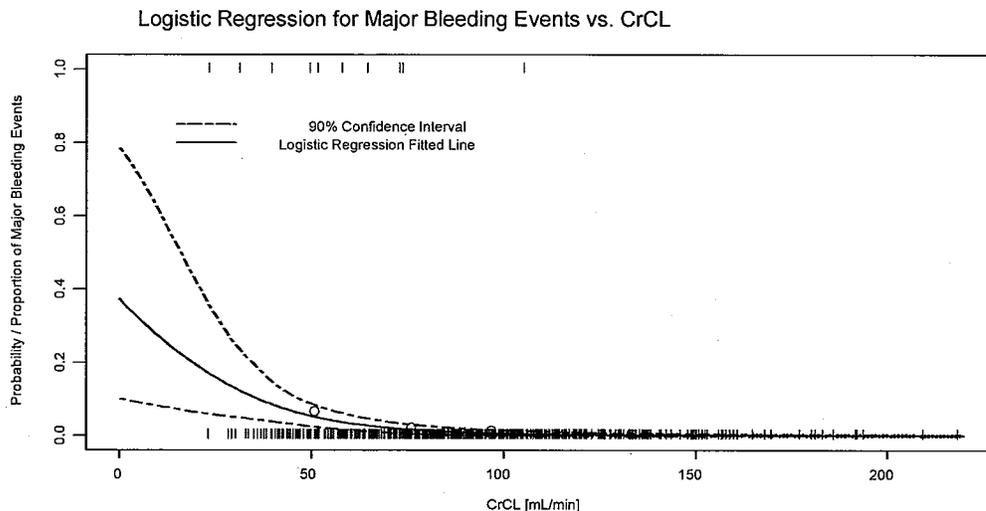
The main safety concern for enoxaparin is major hemorrhage, including a clinically overt hemorrhage resulting in a fall of ≥ 3 gm/dL in hemoglobin or a retroperitoneal, intracranial, or intraocular hemorrhage. Based on TIMI 11A data (unstable angina and non-Q wave myocardial infarction patient), a univariate logistic regression model was used to relate the probability of major or all hemorrhagic events to patient creatinine clearance (CrCL). An increased trend of all hemorrhagic and major hemorrhagic events can be seen while the creatinine clearance decreases and a significant creatinine clearance effect is demonstrated ($P < 0.05$) (Fig 2 and Fig3). It is to note that this analysis does not account for exposure differences. The model predicts percentages of major hemorrhage for patient with creatinine clearance from 80 to 90 mL/min (the threshold for creatinine impaired patient) is about 1%. The value increases to about 3-5% for major bleeding events, when creatinine clearance dropped to 50-60 mL/min (threshold to moderate renal impairment). 12% of major bleeding events can be seen when the creatinine clearance value further dropped to 30mL/min (threshold to severe renal impairment). Further reducing creatinine clearance value to 20 mL/min yields the percentage of major bleeding close to 20%. There is about 10-20, 5-10, 2-5 fold increase in the risk of major hemorrhage for patients with severe, moderate and mild renal impairment respectively. As shown in Fig 3, the slope of the risk for major hemorrhage becomes steep for patients with compromised renal function. The results demonstrated that patients with a decreased creatinine clearance tend to experience more major hemorrhagic episodes.

Fig2. Logistic Regression for All Bleeding Events vs. Creatinine Clearance (CrCL) following 30 mg iv.bolus, followed by 1-1.25 mg/kg SC. maintenance dose



Note: Predicted probability (lines) is generated using all 448 subject data. The open cycle dots are the summary probability from the 448 subjects to evaluate goodness of fit. The bars are the observed events. (0 means patient did not have all hemorrhagic events, whereas 1 means patient had).

Fig 3. Logistic Regression for Major Bleeding Events vs. Creatinine Clearance (CrCL) following 30 mg i.v. bolus, followed by 1-1.25 mg/kg SC. Maintenance dose



Note: Predicted probability (lines) is generated using all 448 subject data. The open circle dots are the summary probability from the 448 subjects to evaluate goodness of fit. The bars are the observed events. (0 means patient did not have major hemorrhagic events, whereas 1 means patient had).

In an effort to investigate the enoxaparin exposure and patient covariate effects on the risk of major hemorrhage, a multivariate logistic model was explored. A stepwise selection was conducted to screen the effects from enoxaparin exposure (AUC), patient age, creatinine clearance, gender, and body weight. Only enoxaparin exposure and patient creatinine clearance were chosen in the final model as significant effects ($P < 0.05$). Based on the model prediction, for patients with the same median exposure levels at steady state AUC 0-12hr of 12 IU*hr/mL, patients with 30 mL/min, 50 mL/min, and 80 mL/min creatinine clearance exhibit respective risks of major hemorrhagic events of 7.6%, 3.6%, and 1.1%. The results suggest that the patients with lower creatinine clearances independent of exposures tend to have increased risk for major hemorrhage.

The combined effect of enoxaparin exposure and patient creatinine clearance on the risk of major hemorrhage is illustrated in the Fig 4. A general trend can be seen that a patient with a higher enoxaparin exposure and/or lower creatinine clearance always incurs a higher risk for major hemorrhage. However, the slope changes remarkably at different exposure and creatinine clearance regions. For example, a patient with creatinine clearance of 100 mL/min and at the enoxaparin daily steady state AUC of 17 IU*hr/mL (equivalent to the 10th percentile of the level observed in the TIMI 11A study), the risk for major hemorrhage is about 0.2%. Even if the level is doubled, and creatinine clearance is decreased by 20%, the risk of major hemorrhage is still only about 0.9%. Comparing to a person with creatinine clearance of 50 mL/min at the AUC value of 37 IU*hr/mL, the correspondent risk of major hemorrhage is 6.6%. A slight increasing of AUC value to 40 IU*hr/mL and decreasing the creatinine clearance value to 45 mL/min,

increases the predicted risk to about 10%. The plot also demonstrates that the risk of major hemorrhage is low and the trend is flat when the creatinine clearance is high, irrespective of the enoxaparin exposure. It suggests that regardless of enoxaparin exposure, patients with high creatinine clearance are least likely to suffer from major hemorrhage. Therefore, the dose adjustment for this patient group is not needed. Nevertheless, a high risk of major bleeding is evident for patients with low creatinine clearance and high enoxaparin exposure. A slight increase in enoxaparin exposure and a decrease in creatinine clearance lead to a remarkable shift in the risk of major hemorrhage. Thus, the dosing adjustment appears to be essential for this patient population. Since the final multivariate logistic model indicates that the risk of major hemorrhage is a function of both creatinine clearance and enoxaparin exposure, reduction of enoxaparin exposure can be used to compensate the loss of renal function.

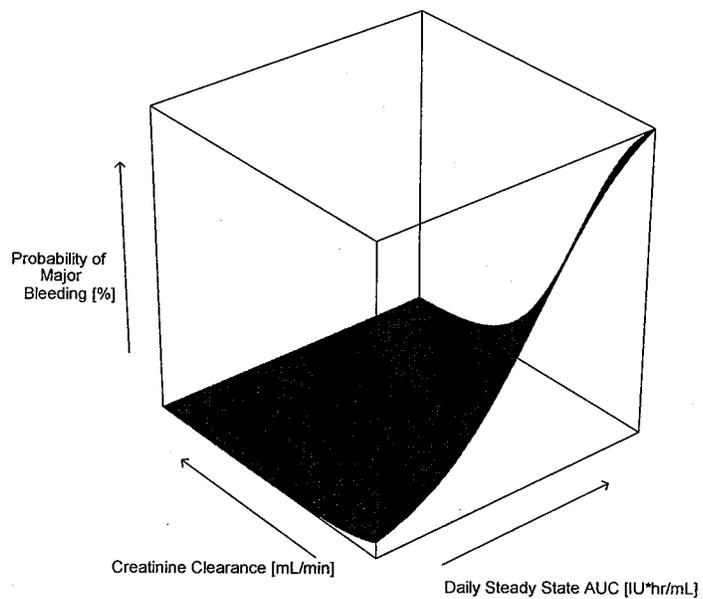
Combined the enoxaparin population PK model and exposure-major hemorrhage model, dose reduction is needed to avoid over-expose the patient and reduce the risk of major hemorrhage in patients with impaired renal functions. The sponsor used 30mL/min (threshold for severe renal impairment patient) as a cut-off point and patients with creatinine clearance \leq 30mL/min will be given 1mg/kg enoxaparin q 24hr rather than q 12hr. Following this regimen, the simulated probability of major hemorrhage was plotted in Fig 5. The severe renal impairment patients belong to the high-risk group for major hemorrhage, since low creatinine clearance and high enoxaparin exposure are both expected. With no dosing adjustment, the expected median probability of major hemorrhage can be as high as to about 40%. Under suggested dosing adjustment, the risk could be reduced to less than 20%. Alternative dosing regimens with creatinine clearance cut-off points of 80mL/min (threshold for mild renal impairment), or 50mL/min (threshold for moderate renal impairment) were also evaluated through the model. Using these cut-off values, the highest risk patient population will not obtain additional benefit from them; however the mild and moderate renal impairment patient might obtain some improvement for their risks of major hemorrhage.

In summary, the dose reduction is necessary for patients with reduced creatinine clearance. Dosing regimen changes from 1mg/kg every 12 hr to 1mg/kg every 24 hr is able to substantially reduce the incidence of major hemorrhage in the high-risk patient population whose creatinine clearance is lower than 30mL/min. This dosing regimen is reasonable. It is to note that this dose adjustment is purely based on the safety signal. The change in efficacy due to dose reduction is not evaluated since no clear exposure-effectiveness relationship is available.

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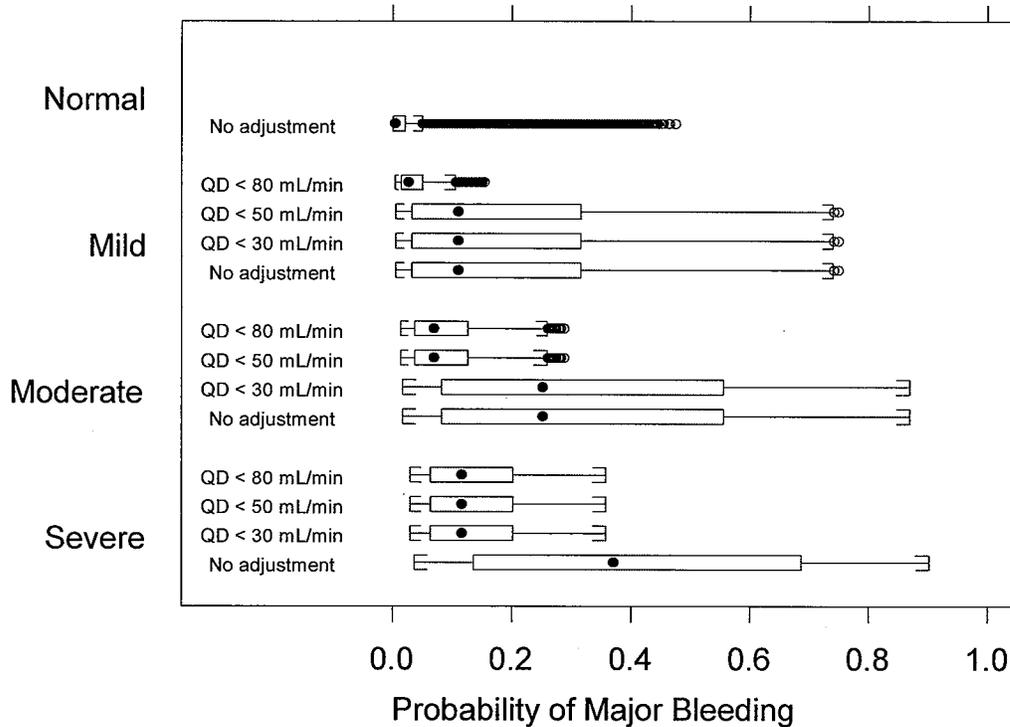
Fig 4. The combination effect of enoxaparin exposure and creatinine clearance on the risk of major hemorrhagic events.

Probability of Major Bleeding vs. CLCR and AUC (Unadjusted Dose)



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Fig 5. Probability of major hemorrhage in normal or renal impairment patient in unadjusted dosing regimen and alternative dosing regimen



Note:

Labels on the y axis represent different patient groups:

1. Normal = patients with creatinine clearance (CrCL) > 80mL/min,
2. Mild = patients with CrCL (50mL/min - 80mL/min),
3. Moderate = patients with CrCL (30mL/min - 50mL/min),
4. Severe = patients with CrCL < 30mL/min.

Labels next to each box plot represent the suggested dose-adjustment.

1. No adjustment = patients were given 1mg/kg maintenance dose q12hr, regardless of their renal functions.
2. QD < 30mL/min = patients were given 1mg/kg q24hr when their CrCL < 30mL/min, otherwise patients were given 1mg/kg q12hr as maintenance dose.
3. QD < 50mL/min = patients were given 1mg/kg q 24hr when their CrCL < 50mL/min, otherwise patients were given 1mg/kg q 12hr as maintenance dose.
4. QD < 80mL/min = patients were given 1mg/kg q24 hr when their CrCL < 80mL/min, Otherwise patients were given 1mg/kg q 12hr as maintenance dose.

3. Recommendations

The Office of Clinical Pharmacology finds that the sNDA is acceptable.

The dosing adjustment based on renal clearance is necessary and sponsor using 1mg/kg q 24 hr instead of q 12 hr as maintenance dose for patients with severe renal impairment is acceptable.

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