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Add the following:

▲Bivalirudin for Injection

DEFINITION

Bivalirudin for Injection is a sterile, lyophilized powder or cake for reconstitution. Bivalirudin used in the manufacture of Bivalirudin for Injection complies with the compendial requirements stated in the Bivalirudin monograph. It contains the equivalent of NLT 90.0% and NMT 110% of the labeled amount of bivalirudin ($C_{98}H_{138}N_{24}O_{33}$).

IDENTIFICATION

• A.

Standard solution and Sample solution: Prepare as directed in the Assay.

Identity sample solution: Mix equal volumes of the *Standard solution* and *Sample solution*.

Acceptance criteria: The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the Assay. The major peaks of the *Identity sample solution* co-elute.

• B. BIOIDENTITY

Thrombin inhibition activity

Buffer solution: 50 mM [tris\(hydroxymethyl\)aminomethane hydrochloride](#) and 120 mM [sodium chloride](#) in [water](#). Adjust to a pH of 7.40 ± 0.04. Add [bovine serum albumin](#) to this solution to obtain a 1-mg/mL concentration and pass through a filter of 0.45-µm pore size.

Stop solution: [Glacial acetic acid](#)

Chromogenic substrate solution: 5 mM solution of *H*-D-cyclohexylalanyl-Ala-Arg-*p*-nitroanilide diacetate salt in [water](#)

Human thrombin solution: 10 µg/mL of human thrombin¹ in *Buffer solution*

Diluted human thrombin solution: Dilute *Human thrombin solution* with *Buffer solution* to obtain a 0.5-µg/mL solution. Thrombin from alternate sources must be standardized in the substrate reaction. To perform the standardization, prepare 6 replicates by adding 910 µL of *Buffer solution* to each sample tube followed by 30 µL of *Chromogenic substrate solution*. Add 60 µL of *Diluted human thrombin solution*, mix on a vortex mixer, and begin the 20-min incubation, as described below. Determine the absorbance at 405 nm for each replicate. Use [water](#) to auto-zero.

Calculate the standardized volume (E_c) of *Diluted human thrombin solution* from the mean absorbance (A_M) as follows:

$$E_c = (0.45 \times 60 \text{ }\mu\text{L})/A_M$$

Standard solution: Prepare a 0.6-mg/mL solution of [USP Bivalirudin RS](#) in [water](#). Dilute with *Buffer solution* to obtain a 5-µg/mL solution.

Sample solution: Prepare a 0.6-mg/mL solution of Bivalirudin for Injection in [water](#). Make 3 independent preparations of this solution. Measure the absorbance of each of the 0.6-mg/mL solutions at 275 nm (A_{275}), using [water](#) to auto-zero.

Calculate the concentration of bivalirudin ($C_{98}H_{138}N_{24}O_{33}$), $C_{B'}$ in mg/mL, in the *Sample solution*:

$$C_B = A_{275}/0.62$$

From this concentration, dilute with *Buffer solution* to obtain a 5-µg/mL solution. Prepare triplicate dilutions from each independently prepared 0.6-mg/mL solution.

Blank, Control test solution, Sample test solution, and Standard test solution

Analysis: In each sample tube, add the *Buffer solution* first, then 30 µL of *Chromogenic substrate solution*, and then the *Standard solution* or *Sample solution* (if using). Mix on a vortex mixer and incubate for 10 min at 37° in a water bath. Add the appropriate volume (E_c) of *Diluted human thrombin solution* to give a final concentration of 0.095 NIH Units/mL and activate the chronometer immediately. Mix on a vortex mixer for a few seconds and heat to 37° for 20 min ± 15 s in a water bath. Stop the reaction by adding 100 µL of *Stop solution*. Measure the absorbance of the 6 solutions at 405 nm using [water](#) to auto-zero. Prepare the solutions for the analysis as indicated in [Table 1](#).

Table 1

	Blank	Control Test Solution	Sample Test Solution	Standard Test Solution
Number of replicates	1	6	3	3



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	Blank	Control Test Solution	Sample Test Solution	Standard Test Solution
Total number of UV readings	1	6	9	3
Chromogenic substrate solution	30 µL	30 µL	30 µL	30 µL
Bivalirudin	100 µL (<i>Standard solution</i>)	0	100 µL (<i>Sample solution</i>)	100 µL (<i>Standard solution</i>)
Diluted human thrombin solution	0	E_c	E_c	E_c
Buffer solution	1000 µL – 30 µL – 100 µL	1000 µL – 30 µL – E_c	1000 µL – 30 µL – 100 µL – E_c	1000 µL – 30 µL – 100 µL – E_c

System suitability**Samples:** *Control test solution* and *Standard test solution***Suitability requirements****Relative standard deviation:** NMT 5% for 6 replicates, *Control test solution***Mean absorbance:** Between 0.428 and 0.473, *Control test solution***Average inhibition:** 44%–50%, *Standard test solution***Analysis****Samples:** *Control test solution* and *Sample test solution*

Calculate the percentage of thrombin inhibition for each of the 9 sample readings of the sample tested:

$$\text{Result} = [1 - (r_u/r_s)] \times 100$$

 r_u = absorbance response from the *Sample test solution* r_s = average absorbance response from the 6 readings of the *Control test solution*

The final percentage inhibition result is given as the average of these 9 values.

Acceptance criteria**Average inhibition:** 42%–52%**Inhibition of each single Sample test solution:** 41%–53%**Relative standard deviation of the inhibition of the Sample test solutions:** NMT 10%, 9 readings**ASSAY****• PROCEDURE****Buffer solution:** Dissolve 8.2 g of [sodium acetate](#) in 900 mL of [water](#). Adjust with [glacial acetic acid](#) to a pH of 6.5 ± 0.1 . Dilute with [water](#) to 1000 mL and pass through a filter of 0.2-µm pore size.**Solution A:** *Buffer solution* and [water](#) (1:1)**Solution B:** *Buffer solution* and [acetonitrile](#) (1:1)**Mobile phase:** See [Table 2](#).**Table 2**

Time (min)	Solution A (%)	Solution B (%)
0	90	10
5	85	15
30	65	35
35	65	35
35.1	90	10
40	90	

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