Modified Plasma-Based Ecarin Clotting Time Assay for Monitoring of Recombinant Hirudin During Cardiac Surgery

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Abstract

Recombinant hirudin (r-hirudin) is being used increasingly for therapeutic anticoagulation in patients with heparin-induced thrombocytopenia undergoing cardiovascular surgery. Although multiple laboratory methods are available for measuring r-hirudin, the ecarin clotting time (ECT) is the most commonly used for this purpose. Ecarin (extracted from snake venom) converts prothrombin to meizothrombin, which promotes clot formation. Direct thrombin inhibitors, like r-hirudin, bind meizothrombin and yield a linear, dose-dependent prolongation of ECT. Low levels of prothrombin and fibrinogen in plasma samples can lead to higher ECT, suggesting falsely elevated r-hirudin levels. A modified ECT assay with prothrombin and fibrinogen in excess was optimized using an orthogonal array method to eliminate the variations in patients’ plasma prothrombin and fibrinogen levels for accurate determinations of plasma r-hirudin levels. By using the modified ECT assay, falsely elevated r-hirudin levels can be avoided in patients undergoing cardiopulmonary bypass, thus providing reliable and accurate r-hirudin monitoring in this clinical setting.

Recombinant hirudin (r-hirudin) is a potent thrombin inhibitor that has been used successfully as an antithrombotic agent in patients with acute coronary syndromes and in the treatment and prevention of venous thrombosis.1-6 Recombinant hirudin has been particularly useful as an alternative to heparin in patients with heparin-induced thrombocytopenia who require further parenteral anticoagulation or need urgent cardiac surgery.7-10

Accurate monitoring of the r-hirudin plasma levels is essential for patient safety. The therapeutic window of r-hirudin is narrow, and there is a high interindividual variability in r-hirudin plasma levels, even when infusion of r-hirudin is adjusted to body weight.3,4,11,12 As a result, patients are at increased risk of inadequate anticoagulation (occlusion of bypass circuit) or excessive bleeding when a fixed-dose protocol of r-hirudin is used in lieu of titrating r-hirudin to targeted plasma levels during cardiac surgery.

Several assays are available for monitoring hirudin levels, but each has its limitations. Although the activated clotting time is used commonly in cardiac surgery, studies of ex vivo blood samples from patients undergoing cardiopulmonary bypass (CPB) and in a dog model of CPB found this unsuitable for monitoring r-hirudin levels.11,13 Activated partial thromboplastin time (aPTT) initially was used for determining r-hirudin activity in several phase I studies.1,2,6,12,14 Subsequent studies, however, demonstrated that large interindividual variability of the aPTT and plasma levels of r-hirudin preclude its use in the setting of cardiac surgery.12,15,16

The thrombin-based chromogenic substrate assay can provide an accurate determination of hirudin plasma levels for a wide range of r-hirudin concentrations.17-19 The ecarin clotting time (ECT) assay was developed based on the unique ability of ecarin
to generate meizothrombin from prothrombin. Meizothrombin immediately binds r-hirudin as it is formed and is unable to promote clot formation until its concentration exceeds that of r-hirudin. Meizothrombin not neutralized by r-hirudin can induce the conversion of fibrinogen to fibrin. 

Prothrombin Reagent

Lyophilized prothrombin (10 U/vial; Sigma-Aldrich, St Louis, MO) was reconstituted with deionized water, and the prothrombin reagent was used to obtain different final prothrombin concentrations in the ECT assay. The prothrombin was diluted with deionized water rather than pooled human plasma because the use of plasma induced enormous clot formation rapidly owing to trace amounts of factor Xa and factor Va in the pooled human plasma.

Materials and Methods

Blood Samples

Pooled plasma samples (n = 60) were obtained from laboratory samples with normal prothrombin times and aPTTs. Blood samples were collected into 0.11 mol/L of sodium citrate (9:1, blood/citrate), and plasma samples were prepared by centrifugation at 3,000g for 8 minutes. Plasma samples were stored in aliquots of 1 mL at −20°C until used. Prothrombin and fibrinogen concentrations were determined, and the pooled plasma was diluted with normal saline to obtain different concentrations of prothrombin and fibrinogen.

Lepirudin (Refludan)

Lepirudin (Refludan, Hoechst Marion Roussel, Kansas City, MO), a recombinant hirudin derived from yeast cells, is a single-chain polypeptide of 65 amino acids (molecular weight, 6979.5 d). Natural hirudin is produced in trace amounts as a family of highly homologous isopolypeptides by the leech Hirudin medicinalis. One molecule of lepirudin binds to one molecule of thrombin and thereby blocks the thrombogenic activity of thrombin. As a result, all thrombin-catalyzed procoagulant reactions, such as conversion of fibrinogen to fibrin; activation of coagulation factors V, VIII, and XIII; and thrombin-induced platelet activation were inhibited. Normal saline (0.9%) was used to reconstitute and dilute r-hirudin.

Fibrinogen Reagent

Lyophilized fibrinogen (Sigma-Aldrich) was dissolved by dispersing the fibrinogen onto 37°C prewarmed 0.9% normal saline with gentle agitation to prevent the formation of small aggregates. Once the aggregates were formed, they were difficult to redisolve. Vigorous mixing of the fibrinogen solution was avoided since this process would break the fibrinogen molecules and make them dysfunctional. The manufacturer’s suggested maximum solubility was 20 mg/mL. The desired amount of clottable fibrinogen in the solution was estimated by using the formula:

\[ \text{Clottable Fibrinogen} = \text{Weight of Fibrinogen} \times \text{Protein by Biuret} \times \text{Clottable Protein} \]

The information for protein by biuret and clottable protein was obtained from the manufacturer’s certificate of analysis. The final concentration of the dissolved fibrinogen was measured based on the Clauss method.

Ecarin

Ecarin was obtained from Sigma-Aldrich; 1 vial contained 50 to 54 ecarin units (EU). One EU activated prothrombin to produce 1 unit of amidolytic activity at pH 8.4 at 37°C. The vial of ecarin was reconstituted with 7.0 to 10.0 mL of 0.05 mol/L of tris(hydroxymethyl)aminomethane buffer containing 0.025 mol/L of calcium chloride, resulting in a stock concentration of 7.7 to 5.3 EU/mL. The ecarin solution was divided into aliquots and stored frozen at −80°C. As an alternative, the ecarin solution could be stored at 4°C for up to 7 days without loss of activity (personal observation).

M-ECT Assay

The M-ECT assay was performed automatically with the AMAX 190 coagulation analyzer (Sigma-Aldrich). We added 25 µL of the prothrombin reagent to 100 µL of the plasma sample, followed by 25 µL of the fibrinogen reagent, which then was incubated at 37°C. After incubation for the desired time, 25 µL of the ecarin reagent was added to the sample, and the clotting time was recorded.
The sequential addition of prothrombin and fibrinogen reagents before the addition of ecarin was essential because an instantaneous clot would form if prothrombin and fibrinogen solutions were mixed together as a single reagent solution. This enabled the prothrombin and fibrinogen solutions to be used as reagents to provide excess coagulant factors without the problem of the reagents clotting.

Optimization of Prothrombin, Fibrinogen, and Ecarin Concentrations and Incubation Time Using an Orthogonal Array

At least 4 parameters needed to be optimized, ie, the concentrations of prothrombin, fibrinogen, and ecarin and the incubation time. The experimental setup used an orthogonal array to minimize the number of actual experimental runs required to obtain useful information about the individual contributions of the control parameters on the desired outcome. The L₉ (3⁴) was a simple orthogonal array designed for studying up to 4 control factors. Table 1 and Table 2 show the experimental design using the L₉ (3⁴) array for optimization of the M-ECT assay.

Calibration Curve for the ECT Assay

A calibration curve was constructed by using citrated-anticoagulated plasma spiked with r-hirudin to achieve final concentrations (equivalent to patient plasma samples) of r-hirudin of 0.75, 1.5, 2.0, 3.0, 4.5, and 6.0 µg/mL in 100 µL of plasma. Each r-hirudin concentration was run in duplicate. From the calibration study, method linearity and its reportable range were determined.

Reproducibility of the ECT Assay

To estimate the within-run and run-to-run coefficient of variation (CV), pooled normal plasma samples were spiked with r-hirudin to achieve plasma concentrations of r-hirudin of 2.0 and 4.0 µg/mL in 100-µL plasma samples. The within-run CV was calculated from the results measured 10 times. The run-to-run CVs were calculated from the results measured on 3 different days using the same pool of plasma samples. For each r-hirudin concentration, the ECT determination was performed 6 times each day.

Statistical Analysis

The means of duplicate measurements of M-ECT were obtained. Least squares linear regression analysis was used for the calibration study. Recovery studies were analyzed with an error of less than 10% considered clinically acceptable.
Results

Trials of Reagent Volumes and Optimization of Prothrombin, Fibrinogen, and Ecarin Concentrations and Incubation Time Using an Orthogonal Array

Based on a study by Potzsch et al,\(^2\) the sample and reagent volume combinations of 100 µL of plasma sample, 25 µL of 1,000% (10.00) prothrombin, 25 µL of 1,400 mg/dL (14.0 g/L) fibrinogen, and 25 µL of 5.3-EU/mL ecarin gave ECTs of 93.5 seconds for a plasma hirudin concentration of 2 µg/mL, 65.5 seconds for 1 µg/mL, and 35.3 seconds for 0 µg/mL. These sample and reagent volume combinations were found to be acceptable for the reaction time measured in seconds without conceivable overlap due to random noise of the measuring system. With the volume held constant, the concentrations of prothrombin, fibrinogen, and ecarin and the incubation time were optimized by using an orthogonal array as outlined in Tables 1 and 2. The optimal combination of a 60-second incubation time and 500% (5.00) prothrombin, 2,320 mg/dL (23.2 g/L) fibrinogen, and 4.0 EU/mL ecarin concentrations was determined. A final higher ecarin concentration of 7.7 EU/mL was used for the M-ECT assay so that a reasonable clotting time (within 100 seconds) was obtained to accommodate the 4 µg/mL of hirudin in plasma of patients undergoing CPB in whom a therapeutic hirudin level of 3.5 to 4.5 µg/mL is desirable.

Independence of Plasma Prothrombin and Fibrinogen Levels

Normal pooled plasma samples were diluted with normal saline and spiked with hirudin to make different plasma concentrations (final plasma concentrations ranged from 87.5% to 37.5%) with a final hirudin concentration of 2 µg/mL. By using the optimal combination of a 60-second incubation time and 500% (5.00) prothrombin, 2,320 mg/dL (23.2 g/L) fibrinogen, and 4.0 EU/mL ecarin concentrations, there was no significant difference among the 4 concentrations of plasma used, indicating that the assay system was independent of the plasma prothrombin and fibrinogen concentrations Table 3.

![Table 3](image)

<table>
<thead>
<tr>
<th>Normal Pooled Plasma With 2 µg/mL of Hirudin (%)</th>
<th>ECT (s)</th>
<th>ECT (s)</th>
<th>Mean ECT (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>87.5</td>
<td>99.4</td>
<td>99.4</td>
<td>99.4</td>
</tr>
<tr>
<td>75</td>
<td>99.8</td>
<td>99.6</td>
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</tr>
<tr>
<td>50</td>
<td>99.5</td>
<td>99.5</td>
<td>99.5</td>
</tr>
<tr>
<td>37.5</td>
<td>99.6</td>
<td>99.6</td>
<td>99.6</td>
</tr>
</tbody>
</table>

ECT, ecarin clotting time; M-ECT, modified ECT.

The optimal combination of a 60-second incubation time, a prothrombin level of 500% (5.00), a fibrinogen level of 2,320 mg/dL (23.2 g/L), and an ecarin level of 4.0 EU/mL was used in this experiment.

Linearity of the M-ECT Assay

The M-ECT assay performed with an ecarin concentration of 7.7 EU/mL was linear from 0 to 6 µg/mL plasma hirudin concentrations with a correlation coefficient \( r = 0.99878 \) Figure 1. At 8 µg/mL of hirudin, the ECT became nonlinear with these assay parameters (data not shown).

Reproducibility of the M-ECT Assay

The assay was highly reproducible with within-run CVs of 2.8% for 2 µg/mL and 3.9% for 4 µg/mL, and the between-run CVs were equivalent to the within-run CVs with 3.2% for 2 µg/mL and 4.6% for 4 µg/mL.

Recovery of the M-ECT Assay

A recovery of 107% was obtained at 1.5 µg/mL, and 92% was obtained at 3 µg/mL.

Discussion

Treatment with r-hirudin has a narrow therapeutic window. Several clinical studies have demonstrated that the risk of r-hirudin–associated bleeding complications is related to the increased plasma concentration of r-hirudin.\(^3,4,28\) Plasma levels of r-hirudin are affected by the dose administered and the individual renal clearance rate. Accurate monitoring of the plasma r-hirudin level is required to avoid bleeding or thromboembolic complications during r-hirudin treatment. Despite initial reports of aPTT providing a reliable r-hirudin monitoring system,\(^1,2\) subsequent studies indicated that the aPTT was not suitable for accurate r-hirudin measurement,\(^12,15\) and high inter-individual variability of aPTT prolongation was found at similar hirudin

![Figure 1](image)

**Figure 1** Calibration curve of quantitative determination of the recombinant-hirudin level using the modified ecarin clotting time (M-ECT). \( y = 18.289 + 10.947x; r = 0.99878. \)
ECT, however, is not affected by plasma concentrations of heparin up to 1.0 U/mL. When complexed with antithrombin, heparin does not inhibit meizothrombin generated by ecarin. Direct antithrombin agents such as hirudin and synthetic low-molecular-weight thrombin inhibitors inhibit meizothrombin directly. Therefore, the heparin-antithrombin complex cannot exert a synergistic influence on the inhibitory action of hirudin on meizothrombin.

We developed a modified plasma-based ECT assay by adding prothrombin and fibrinogen in excess to the patient’s plasma sample to alleviate the conventional ECT’s dependence on plasma levels of prothrombin and fibrinogen, especially in high plasma r-hirudin levels. In addition, we optimized the prothrombin, fibrinogen, and ecarin concentrations and the incubation time by using orthogonal array techniques. Our modified ECT assay system has a high degree of accuracy and precision for a wide measuring range, and the ECT method has been reported to be insensitive to hirudin and oral anticoagulants. The modified ECT assay can be performed easily using widely available clot-based coagulation instruments. The analytical turnaround time for the M-ECT was about 13 minutes, given the 8 minutes for patient sample preparation and a maximum of 3 minutes allowed for clot formation in the M-ECT. Thus, the total turnaround time from the time the sample is obtained in the operating room, including transport and analytic times, to the reporting time would be estimated to be less than 20 minutes.

The applicability of the optimization M-ECT assay will need to be validated using samples for patients receiving hirudin during cardiac surgery. The potential wide adaptability of the assay would provide clinicians a means to adjust hirudin treatment based on the patient’s plasma hirudin level, especially for patients undergoing cardiac surgery, and, hence, the risks of overtreatment or undertreatment with r-hirudin could be minimized.

References


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