Ecarin Chromogenic Assay – A New Method for Quantitative Determination of Direct Thrombin Inhibitors Like Hirudin

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Key Words

Hirudin · Thrombin inhibitor · Ecarin chromogenic assay · Ecarin clotting time · Argatroban

Abstract

A new sensitive and precise method for quantitative determination of direct thrombin inhibitors is described, the ecarin chromogenic assay (ECA). Ecarin is used as the specific prothrombin-activating principle. The cleavage of a chromogenic substrate by meizothrombin is inhibited in a concentration-dependent fashion by direct thrombin inhibitors. For the ECA, the linear measuring range is about 0.1–3.0 µg hirudin/ml plasma. Coefficients of variations between 2.3 and 4% over the whole concentration range were achieved. The ECA has proved to be more sensitive than the compared tests (ecarin clotting time and a thrombin-based chromogenic assay); a detection limit of 0.011 µg hirudin/ml and a quantitation limit of 0.032 µg hirudin/ml were calculated. The ECA is independent of the variations of the coagulation variables fibrinogen and prothrombin. Neither heparin nor oral anticoagulants interfere with the ECA.

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Introduction

Natural or synthetic direct inhibitors of thrombin, the key enzyme in blood coagulation, have been increasingly used for the prophylaxis and treatment of thromboembolic disorders. Recombinant hirudin, a highly specific, tight-binding thrombin inhibitor, was the first direct thrombin inhibitor introduced. Because of its narrow therapeutic range, clinical monitoring of hirudin is necessary to avoid inadequate anticoagulation due to underdosage and in order to avoid overdosage being accompanied by severe bleeding complications. Different methods for the determination of direct thrombin inhibitors, especially of hirudin, are in use. Global clotting tests like activated partial thromboplastin time (aPTT), thrombin time and variations thereof or activated coagulation time were investigated regarding their suitability in monitoring direct thrombin inhibitors like hirudin [1–5]. Immunological methods and chromogenic assays as well as special coagulation assays, e.g. the ecarin clotting time (ECT), were developed for the monitoring of anticoagulant therapy [6–16]. The suitability of these methods for the determination of hirudin and other direct thrombin inhibitors, their advantages and drawbacks are discussed in detail by Nowak [17] and Hafner et al. [18]. These authors recommend particularly the ECT and also chromogenic substrate assays as suitable for the monitoring of therapy with direct thrombin inhibitors.

Fig. 1. Principle of measurement of ECA compared with ECT.

A new chromogenic assay based on the same principle as the ECT, the ecarin chromogenic assay (ECA), has been developed to preserve the advantages of the ECT and overcome the deficiencies of the methods for determination of direct thrombin inhibitors used so far.

Materials and Methods

The ECA-H HaemoSys® kit for quantitative determination of hirudin in plasma and the ECA-T HaemoSys® kit for quantitative determination of synthetic direct thrombin inhibitors in plasma are commercially available from HaemoSys GmbH, Jena, Germany. The kits consist of human prothrombin containing ECA prothrombin buffer, the ECA-H or ECA-T substrate, containing a specific thrombin substrate, and the ECA ecarin reagent. Additionally, the kits include standards and controls: ECA-H standards, ECA-H controls and ECA-T standards, ECA-T controls, respectively. Pooled plasma of healthy volunteers ($n = 10$) was purchased from DRK-Blutspendedienst, Institut Gera, Gera, Germany. r-Hirudin, Repludin®, was from Schering AG, Berlin, Germany. Argatroban, Novastan®, was from Texas Biotechnology Corp., Houston, Tex., USA. Human factor-II-deficient plasma and ecarin were obtained from Loxo Ltd., Dossenheim, Germany. Unfractionated heparin (International Standard for Heparin, 82/502, 712 IU anti-IIa/ml) was from the National Institute for Biological Standards and Control, Hertfordshire, UK. Human fibrinogen (Pg-, vWF- and Fn-depleted) was purchased from Kordia bv, Leiden, Netherlands. Defibrinated plasma was made by heating pooled plasma for 4 min at 56°C followed by rapid cooling and centrifugation. The fibrinogen content of plasma was determined by conversion of fibrinogen to fibrin using thrombin, and by determination of the protein clot content using the Biuret method according to Ratnoff and Menzie [19].

As an alternative thrombin-based chromogenic test, the hirudin activity assay from Dade Behring (Dade Behring Vertriebs GmbH & Co., Schwalbach, Germany) was used. According to the instructions of the manufacturer, 25 µl plasma sample and 50 µl substrate reagent were mixed. Reaction was started by addition of 250 µl thrombin reagent, and the change in optical density was measured at 405 nm for 1 min. The test was performed at 37°C on a manual 4-channel coagulation analyzer TEChrom IV plus (Teco GmbH, Neufahrn, Germany).

The ECT test was performed according to Nowak and Bucha [20] using a manual coagulometer (CL4; Behnk Elektronik GmbH & Co. KG, Norderstedt, Germany).

The principle of measurement of the ECA is shown in figure 1. Similar to the ECT, the ECA is based on ecarin-induced prothrombin activation. In the ECT test, plasma prothrombin is used, whereas in the ECA, human prothrombin is contained in excessive amounts in the ECA prothrombin buffer HaemoSys. Unlike in the ECT test where the clotting activity of meizothrombin/meizothrombin-desF1 is measured, the amidolytic activity towards a chromogenic substrate is determined in the ECA by the measurement of *p*-nitroaniline release. Changes in optical density are recorded at 405 nm. The time is stopped when a threshold of 0.1 absorption units is reached (ECA reaction time).

Reagents for the ECA were dissolved according to the instructions of the manufacturer. The test procedure for ECA HaemoSys was as follows:

100 µl ECA prothrombin buffer
prewarmed in the coagulation analyzer at 37°C
+ 25 µl plasma sample
room temperature
+ 25 µl ECA substrate
prewarmed in the coagulation analyzer at 37°C
incubation for 1 min at 37°C
+ 50 µl ECA ecarin reagent
start of measurement

For the determination of hirudin in plasma, the ECA-H substrate and ECA-H method were used, whereas for the determination of the synthetic direct thrombin inhibitor argatroban, the ECA-T substrate and ECA-T method were used. ECA was measured using the 2-channel manual coagulation analyzer Coatron M2 and the 4-channel coagulation analyzer TEChrom IV plus (both Teco GmbH, adapted for ECA-H and ECA-T), respectively.

Results

To prepare an ECA reference curve, pooled plasma of healthy volunteers was spiked with known amounts of hirudin. An example curve for the 2-channel coagulation analyzer Coatron M2 is given in figure 2. The reference

Fig. 2. Reference curve of quantitative determination of hirudin using ECA. Data were acquired on Coatron M2; n = 30, mean values and SD are given.

Table 1. Imprecision data of ECA compared with a thrombin-based chromogenic test and ECT

	Added amount of hirudin, µg/ml					
	0.062	0.125	0.250	0.500	1.000	2.000
Number of replicates	30	30	30	30	30	30
<i>Thrombin-based chromogenic test</i>						
Mean, µg/ml			0.213	0.504	1.048	1.980
SD, µg/ml			0.031	0.042	0.070	0.094
CV, %			14.4	8.4	6.7	4.8
Recovery, %			85.2	100.8	104.8	99.0
Detection limit, µg/ml	0.199					
Quantitation limit, µg/ml	0.575					
<i>ECT test</i>						
Mean, µg/ml	0.073	0.133	0.269	0.543	1.088	2.080
SD, µg/ml	0.012	0.017	0.023	0.042	0.067	0.111
CV, %	16.7	12.8	8.6	7.7	6.1	5.3
Recovery, %	117.7	106.4	107.6	108.6	108.8	104.0
Detection limit, µg/ml	0.046					
Quantitation limit, µg/ml	0.138					
<i>ECA</i>						
Mean, µg/ml	0.063	0.126	0.232	0.503	1.020	1.991
SD, µg/ml	0.004	0.005	0.009	0.016	0.033	0.045
CV, %	6.2	4.0	3.9	3.2	3.2	2.3
Recovery, %	101.6	100.8	92.8	100.6	102.0	99.6
Detection limit, µg/ml	0.011					
Quantitation limit, µg/ml	0.032					

Hirudin was determined using a thrombin-based chromogenic test on TEChrom IV plus coagulation analyzer, ECT test on CL4 coagulometer, and ECA on TEChrom IV plus coagulation analyzer.

Fig. 3. Reproducibility of ECA. Data were acquired on Coatron M2; values of reference curves determined on 24 different days using 6 different reagent batches (○) and the mean values of it with SD (●).

Fig. 4. Stability of ECA reagents in use. Data were acquired on TEChrom IV plus; mean values and SD of 5 different sets of reagents, ECA prothrombin buffer, ECA-H substrate and ECA ecarin reagent are given.

curve shows a clear linearity between ECA reaction time and inhibitor concentration over a broad concentration range. Linearity was observed at up to 3 µg/ml (data not shown). Imprecision data for hirudin determination in plasma using the manual 4-channel coagulation analyzer TEChrom IV plus are given in table 1. Data calculated for the ECA are compared with those obtained by the thrombin-based chromogenic assay using the same coagulation analyzer and ECT, respectively. Accuracy is ex-

pressed as percent recovery. The detection limit is expressed as $3.3 \times \text{SD}/S$, the quantitation limit as $10 \times \text{SD}/S$, where SD represents the standard deviation of the blank ($n = 30$) given in seconds and S represents the slope of the reference curve. Using ECA, coefficients of variation (CV) between 2.3 and 4.0%, and 6.2% for the low plasma hirudin concentration of 0.062 µg/ml, were achieved. With ECT, CV between 5.3 and 8.6%, and in the lower range 12.8% for 0.125 µg/ml and 16.7% for

Fig. 5. Influence of the plasma prothrombin level on hirudin determination using ECA (a) and ECT (b). Data were acquired on TEChrom IV plus; n = 3.

0.062 µg/ml, were determined. Using the thrombin-based chromogenic assay, CV between 4.8 and 14.4% were obtained. For this test, a detection limit of 0.199 µg/ml was calculated; therefore, the hirudin concentrations of 0.125 and 0.062 µg/ml were not measured (table 1).

The reproducibility of ECA was determined by measuring hirudin reference curves on 24 days within 2 months using different batches of reagents. Figure 3 shows that there is a very good day-to-day and inter-batch reproducibility.

The stability of the ECA reagents in use is shown in figure 4. Five sets of reagents (ECA prothrombin buffer, ECA-H substrate and ECA ecarin reagent) were tested on 12 different days. Reagents were stored at 37°C in the coagulation analyzer for the time of measurement, at least for 3 h daily and at 4–8°C in the intermediate time. Figure 4 shows that the reagents were stable after dissolution for 11 days, whereas on day 12, the measured hirudin concentrations decreased.

The influence of the plasma prothrombin level on ECA and ECT was investigated; results are given in figure 5. Plasma containing a prothrombin level of about 200% was prepared by addition of 80 µg of human prothrombin to 1 ml of pooled plasma; plasma containing prothrombin levels between 0 and 75% was prepared using factor-II-deficient plasma and mixtures thereof with pooled plas-

ma. The samples were spiked with different amounts of hirudin. The ECA was shown to be absolutely independent of the plasma prothrombin level and can be carried out even in prothrombin-deficient plasma. The ECT test was impracticable in prothrombin-deficient plasma samples. Even plasma prothrombin levels of less than 75% resulted in the prolongation of the ECT and falsely high hirudin concentrations. The error increases with increasing hirudin levels (fig. 5).

Figure 6 shows the influence of plasma fibrinogen content on ECA and ECT, respectively. Heat-defibrinated plasma and mixtures thereof with pooled plasma were used. An elevated fibrinogen level of about 300% (8.9 g/l fibrinogen measured) was prepared by adding human fibrinogen to pooled plasma. Using different amounts of hirudin, no influence of the fibrinogen level on ECA and only a moderate influence on ECT could be observed. In fibrinogen-enriched plasma, there is a decrease in hirudin recovery of about 20%, and in plasma with a fibrinogen content of 25% of the normal pooled plasma, there is an increase in hirudin recovery of about 30% using ECT. Due to its principle of measurement, the ECT test was impracticable in fibrinogen-free plasma samples.

The usability of the ECA for the determination of direct thrombin inhibitors other than hirudin was tested

Fig. 6. Influence of the plasma fibrinogen level on hirudin determination using ECA (a) and ECT (b). Data were acquired on TECHrom IV plus; n = 3.

Fig. 7. Reference curve of quantitative determination of argatroban using ECA. Data were acquired on Coatron M2; n = 30, mean values and SD are given.

using the small synthetic inhibitor argatroban. For the reference curve, pooled human plasma was spiked with different amounts of argatroban and measured using the ECA-T method (fig. 7). Imprecision data are given in table 2. There is a clear linear correlation between argatro-

ban concentrations tested and ECA reaction time. The accuracy reported as percent recovery is very high in the concentration range between 0.5 and 2 µg/ml, and becomes somewhat lower at argatroban concentrations below 0.5 µg/ml.

Table 2. Imprecision data of ECA on Coatron M2 2-channel coagulation analyzer for argatroban determination in plasma

	Added amount of argatroban, µg/ml					
	0.062	0.125	0.250	0.500	1.000	2.000
Number of replicates	30	30	30	30	30	30
Mean, µg/ml	0.075	0.119	0.291	0.513	0.986	2.009
SD, µg/ml	0.0067	0.0076	0.0107	0.0172	0.0384	0.0626
CV, %	9.0	6.4	3.7	3.4	3.9	3.1
Minimum, µg/ml	0.064	0.106	0.272	0.485	0.910	1.920
Maximum, µg/ml	0.089	0.135	0.311	0.549	1.048	2.183
Recovery, %	119.3	95.2	116.4	102.6	98.6	100.5
Detection limit, µg/ml	0.020					
Quantitation limit, µg/ml	0.061					

Discussion

For clinical monitoring of hirudin, the manufacturers recommend aPTT. However, it has been shown by many investigators that aPTT is not suited for quantitative hirudin determination because all aPTT assays become less responsive at increasing hirudin concentrations; therefore, toxic blood levels cannot be detected or prevented by measuring aPTT. Further limitations are the dependence of aPTT on the reagent used and the high interindividual variations of the clotting time values [1, 2, 17, 18].

From the variety of methods tested regarding their suitability for monitoring hirudin or other direct thrombin inhibitors, ECT and chromogenic substrate-based assays turned out as tests which can represent the clinical situation more adequately than aPTT [17, 18]. Especially the ECT test is considered as a fast, highly sensitive and precise method allowing hirudin determination over a wide concentration range with low interindividual variations [20].

The chromogenic substrate assays for hirudin determination described so far are based on the inhibition of thrombin, added in excess to the diluted sample, by hirudin. Residual thrombin is determined by measuring its amidolytic activity towards a chromogenic substrate [9, 11–13]. The ECT and our new chromogenic assay ECA are based on meizothrombin generation from prothrombin by ecarin, a snake venom metalloprotease from *Echis carinatus* [16, 21, 22]. Direct thrombin inhibitors like hirudin immediately bind to the permanently formed meizothrombin in a concentration-dependent fashion [23]. In the ECT test, the proteolytic activity of noninhibited generated meizothrombin is quantified by measuring the conversion of fibrinogen to fibrin, i.e. the clotting

time. In the ECA, the amidolytic activity of meizothrombin towards a chromogenic substrate is determined. The comparison of both meizothrombin-generation-based tests ECT and ECA with the thrombin-based chromogenic assay shows that both tests, ECT and even more ECA, are superior to the thrombin-based chromogenic assay regarding sensitivity and precision at hirudin concentrations in the lower range. The sensitivity of the thrombin-based chromogenic assay is insufficient at low hirudin concentrations. It can be improved by increasing the sample volume, but this will result in loss of linearity of the reference curve.

In chromogenic substrate assays for hirudin determination based on the inhibition of added thrombin, heparin or endogenous heparin-like substances can interfere; consequently, a heparin inhibitor has to be added [9, 11–13]. Heparin alone or complexed to antithrombin III cannot inhibit meizothrombin [24]; therefore, ECA as well as ECT are not influenced by heparin.

Limitations of ECT are its dependence on the plasma fibrinogen level as it is a clotting assay and its dependence on the plasma prothrombin level due to the principle of measurement [20, 25]. ECA overcomes both drawbacks. Whereas in the clotting assay ECT, the prothrombin of the plasma sample is used as a substrate for ecarin, in the ECA, human prothrombin is an internal component of the ECA prothrombin buffer. Therefore, the prothrombin level of the plasma sample does not influence the ECA. Because it is not a clotting assay, the fibrinogen content does not influence the ECA.

The ECA kit consists of only three components, their stability allowing to keep them in the coagulation analyzer at 37°C during the measurement, and therefore, incubation times of 30 s or 1 min are sufficient. Because of

the simple test procedure, the ECA can be easily performed both as point-of-care test on manual coagulation analyzers provided with an option for optical measurement and as fully mechanized assay on automated systems [26]. The high level of precision permits each ECA analysis to be carried out as a single determination.

ECA is suitable for quantitative determination of direct thrombin inhibitors other than hirudin. This was shown for argatroban, a synthetic small molecule that selectively inhibits the catalytic site of thrombin in a revers-

ible manner [27]. There is a clear linear relationship between inhibitor concentration and ECA reaction time, which makes the ECA more recommendable for quantitative argatroban determinations than other suggested methods, e.g. aPTT or prothrombinase-induced clotting time [27, 28].

It can be concluded that ECA is a simple, rapid, precise, reliable and specific assay for the quantitative determination of direct thrombin inhibitors that is suitable as a manual or automated diagnostic test.

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