UDC 577.151:612.115 https://doi.org/10.15407/biotech15.03.052

OPTIMIZATION OF THE EVALUATION METHOD OF THE PERFORMANCE OF THERAPY USING INDIRECT ACTION ANTICOAGULANTS

D. S. Korolova[1]
O. V. Hornytska[1]
V. A. Deyev[2]
V. I. Gryshchuk [1]
T. M. Chernyshenko[1]
T. M. Platonova[1]
V. O. Chernyshenko[1]

[1]Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine, Kyiv

[2]Shalimov National Institute of Surgery and Transplantology of the National Academy of Medical Sciences of Ukraine, Kyiv

E-mail: platonovatn@gmail.com

Received 26.04.2022
Revised 17.06.2022
Accepted 30.06.2022

Aim. Treatment by indirect anticoagulants (vitamin K antagonists) requires a personalized approach for controlling the overall level of prothrombin and the accumulation of its decarboxylated forms. The purpose of this work was to optimize the method for monitoring of the therapy with indirect anticoagulants.

Methods. An analysis was performed of 41 blood plasma samples from patients with cardiovascular pathologies. Activated partial thromboplastin time (APTT), prothrombin time, ecamulin time, statistical data analysis ("Statistica 7") have been used.

Results. APTT test allowed identifying the individual sensitivity of patients to indirect anticoagulants. In particular, 20% of patients showed a decrease in the total level of prothrombin, which, together with the accumulation of decarboxylated forms, leads to a risk of bleeding. Individual insensitivity to the action of vitamin K antagonists was determined in 11% of patients.

Conclusion. To control the efficacy of indirect anticoagulants therapy, we developed test with ecamulin (protease from the venom of *Echis multisquamatis*) was used as a prothrombin activator, which can activate not only functionally active prothrombin, but also its decarboxylated forms. Use of ecamulin simultaneously with thromboplastin allows determining in the blood plasma the content of not only functionally active prothrombin, but also the total level of prothrombin, which makes it possible to control the accumulation of decarboxylated prothrombin.

Key words: prothrombin, vitamin K, indirect anticoagulants, thrombolytic therapy.

Currently, four classes of antithrombotic preparations are used clinically for the prevention and treatment of thrombosis: direct anticoagulants (heparin, low molecular weight heparins, inhibitors of thrombin and factor Xa); indirect anticoagulants (inhibitors that affect vitamin K-dependent clotting factors); antiplatelet agents (including non-steroidal anti-inflammatory drugs and clopidogrel that affect platelet adhesion and aggregation); thrombolytic drugs (agents that activate the fibrinolytic system, through the conversion of plasminogen to plasmin) [1–3].

A special place in the prevention of thrombosis belongs to oral anticoagulants, or indirect anticoagulants (IA), which are antagonists of vitamin K. The latter is necessary for the synthesis of functionally active coagulation factors, namely the vitamin K-dependent proteins: prothrombin, factors VII, IX, X, proteins C and S. These factors are key components of the coagulation cascade, and their functional activity determines the hemostatic potential of the blood (Fig. 1). As a result, of impaired post-translational γ-carboxylation of vitamin K-dependent

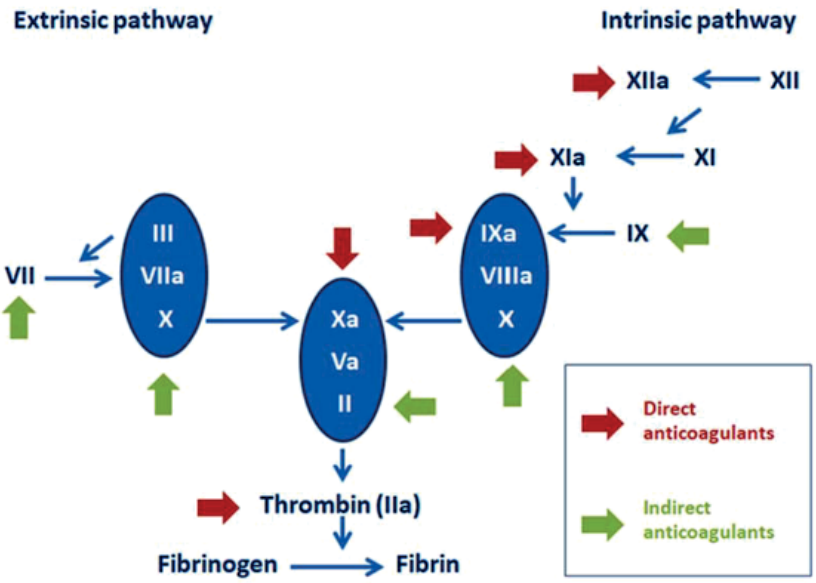

Fig. 1. **Actions of anticoagulants on the factors of the blood coagulation system [8]**
Indirect anticoagulants disrupt the carboxylation of blood coagulation factors, thereby preventing the activation of proenzymes (prothrombin, factor X, factors VII and IX). In contrast, direct anticoagulants act on the active enzymes of the coagulation cascade, predominantly thrombin or factor Xa.

proteins, a number of factors of the blood coagulation system enter the blood in the decarboxylated functionally inactive forms [4, 5]. That leads to a decrease in the procoagulant potential of the blood coagulation system, thereby contributing to the risk of bleeding. This requires constant and systematic determination of the content/activity of vitamin K-dependent factors of the hemostasis system and determines the need for regular monitoring of the degree of hypocoagulation [1, 5–7].

Materials and Methods

APTT-reagent, thromboplastin (thromborel S, Siemens, Germany), $CaCl_2$ solution were purchased from Berichrom. Ecamulin was purified from the venom of *Echis multisquamatis* according to the method of Solovjev et al [8].

Collection of blood plasma of patients. Samples were taken from 41 patients with cardiovascular pathologies (aged 34–80, $n = 41$), who were given warfarin as anticoagulant therapy. Blood collection was performed during anticoagulant therapy. Platelet-poor blood plasma was prepared from citrated blood by centrifugation at 1200 g during 30 min. Sodium Citrate (3.8%) added immediately after collection to the whole blood at 1:9 ratio was used as an anticoagulant [9]. All work was done in accordance with the Declaration of Helsinki. Studies were conducted according to the Ethical Committee Approval No. 8 form 11.05.2018 (Shupyk National Medical Academy of Postgraduate Education of Ministry of Health of Ukraine).

Activated partial thromboplastin time. Activated partial thromboplastin time (APTT) was performed according to the following procedure: 0.1 ml of studied blood plasma was mixed with equal volume of APTT-reagent and incubated during 3 minutes at 37 °C. Then the coagulation was initiated by adding of 0.1 ml of 0.025 M solution of $CaCl_2$ and clotting time was monitored. Time of clotting was evaluated using coagulometer CT2410 (Solar, Belarus).

When clotting time in APTT-test was prolonged we performed the APTT mixing study (inhibitory correction probe) as follows: 0.05 ml of studied blood plasma was mixed with 0.05 ml of control blood plasma sample, 0.1 ml of APTT-reagent and incubated during 3 minutes at 37 °C. Blood clotting was detected as described above. Normalization of blood clotting time indicated the deficiency of the clotting factors, otherwise the accumulation of blood clotting inhibitors was assumed [10].

Mixing study determines if the patient has a factor deficiency or the presence of a factor-inhibiting antibody. Data are interpreted as Dr Castellone indicated in her work: if the plasma of the patient is suspected of being factor deficient, adding the pooled normal plasma will add back the deficient clotting factor,

and the APTT will correct itself. If there is no correction in the corresponding APTT, the plasma of the patient contains an inhibitor, which prevents the ability of the pooled normal plasma to correct itself [11].

Prothrombin time. Thromboplastin reagent (INR = 1.1) was measured as follows: clotting was initiated by mixing 0.1 ml of blood plasma with 0.1 ml of 0.025 M $CaCl_2$ and 0.1 ml of thromboplastin reagent, time of clotting was monitored. Thromboplastin acts through tissue factor pathway of coagulation and activates only carboxylated and uncleaved forms of prothrombin. Time of clotting was evaluated using coagulometer CT2410 (Solar, Belarus). Results of prothrombin test were presented as International normalized ratio (INR) calculated by formula: $INR = (Ap/An)^{isi}$, Ap — studied blood plasma clotting time; An — blood plasma clotting time of healthy control; ISI — international sensitivity index [9].

Ecamulin time. Ecamulin test is based on the application of ecamulin, prothrombin activator from the venom of *Echis multisquamatis*. Ecamulin activates prothrombin, des-gamma-carboxy-prothrombin and prethrombin 1 thus permitting the determination of total prothrombin level [12].

Results of ecamulin test were presented as ecamulin ratio (ER) calculated by formula: ER = Ap/An; Ap — studied blood plasma clotting time; An — blood plasma clotting time of healthy control.

Statistical data analysis. Statistical data analysis was performed using Microsoft Excel. All assays were performed in series of three replicates and the data were fitted with standard errors using "Statistica 7".

Results and Discussion

The state of the blood coagulation system was analyzed in patients ($n = 41$) with cardiovascular pathologies who underwent a course of anticoagulant therapy with IA (warfarin). The state of the blood coagulation system was monitored using the activated partial thromboplastin time (APTT) test. The clotting time of the blood plasma of these patients in the APTT test was increased by 1.5–2.5 times compared with the norm (45 s). APTT mixing test led to the normalization of clotting time, which indicated the accumulation of decarboxylated forms (PIVKA-proteins) of coagulation factors in the blood plasma of patients. To determine the content of functionally active prothrombin, which is a key component of the coagulation cascade, we used the prothrombin time (PT) diagnostic test with thromboplastin as a prothrombin activator. To assess the decarboxylated forms of prothrombin, we used the "ecamulin time" test, a PT test optimized by us, in which ecamulin (analogous to ecarin) was used instead of thromboplastin as an activator of prothrombin. The content of functionally active prothrombin was expressed as a prothrombin ratio (PR); the content of total prothrombin (functionally active and inactive) was expressed as an ecamulin ratio (EO).

Based on the data obtained, the patients were divided into three groups: group 1 — patients with an effectively selected dose of IA (the potential of the coagulation system is reduced by 35–55%), group 2 — patients who are tolerant to IA (the procoagulant potential is fully preserved), group 3 — patients with hypersensitivity to IA (overdose of IA preparations) (Fig. 2).

For patients of the first group, a decrease in PR by 35–55% (0.6) compared with the norm (1.0 ± 0.1) is seen, which indicates the accumulation of decarboxylated prothrombin and a decrease in blood coagulation potential.

In the blood plasma of the second group of patients (Fig. 2), decarboxylated prothrombin, the presence of which is due to vitamin K deficiency, was not detected (ER = PR), which indicates the tolerance to IA.

In patients of the third group, a decrease in functionally active prothrombin to 20–30% was revealed against the background of a low content of total prothrombin (less than 70%). Such a significant decrease in coagulation potential may be accompanied by bleeding, and in such patients the use of IA is contraindicated.

Recent studies show high efficiency of IAs in the prevention and treatment of thrombotic complications and in reducing the risk of thrombosis [13, 14]. However, the individual sensitivity of the patient necessitates selecting the dose of IA preparations and monitoring their performance, which is due both to the characteristics of the patient's condition (age, platelet function, concomitant diseases, nutrition, hypertension, stroke consequences, alcohol dependence), and the problem of compatibility of the drugs used [2, 3, 5].

It should be noted that the total level of prothrombin in the blood plasma of patients can vary between 90–110%. The decrease in the potential of the coagulation system (accumulation of decarboxylated prothrombin forms) must be determined taking into account the total level of prothrombin in the patient in question, and not the average level

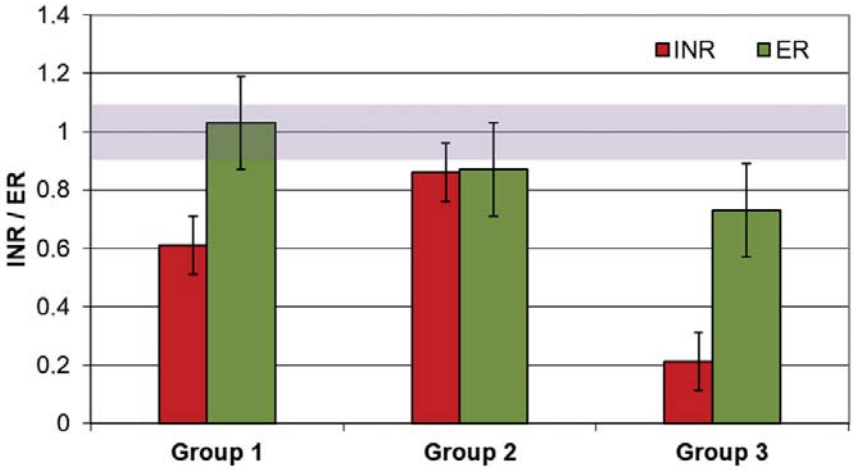

Fig. 2. **Prothrombin (PR) and ecamulin (ER) ratios, obtained from the analysis of blood plasma of patients who underwent IA therapy (peak of the treatment)**
Control (n = 12): INR and ER values are 1.0 ± 0.1

of prothrombin in the blood plasma of donors. The therapeutic interval for the content of functionally active prothrombin in the treatment of IA should be within 30–50%, which corresponds to an INR value of 2–3 [9].

The main control method throughout the clinical use of the IA has been and remains the determination of the prothrombin clotting time of blood plasma (in some cases, of the whole blood). The principle of the method is to determine the clotting time of blood plasma after the addition of thromboplastin in the presence of calcium ions. The test implements a number of successive and interrelated reactions, and the clotting time of blood plasma depends not only on the total rate of the process of activation of coagulation factors, but also on the presence of inhibitors of fibrin polymerization and thrombin inhibitors. Hence, with the obvious simplicity of the test itself, the evaluation of its results is a serious problem that has not been finally resolved to date. In addition, the determination of prothrombin time does not provide information on the presence and content of functionally inactive (decarboxylated) forms of prothrombin, since thromboplastin does not activate them. Therefore, to control the effectiveness of IA preparations, we developed test conditions in which ecamulin (a prothrombin activator from the venom of Echis multisquamatis) was used as a prothrombin activator. Ecamulin, unlike thromboplastin, is able to activate not only functionally active prothrombin, but also its decarboxylated forms [15–17], so the use of this method, which we have optimized, makes it possible to control the total level of prothrombin in blood plasma. Thus, with the parallel use of these two activators, it is possible to determine the content in the blood plasma of not only functionally active prothrombin, but also its decarboxylated forms.

The information content of our optimized method for monitoring the performance of IA therapy is evidenced by the results, on the basis of which the first group of patients was identified. According to the "ecamulin time" test, the content of functionally inactive forms of prothrombin is 40% of the total prothrombin level (Fig. 2). The content of functionally active prothrombin with effective therapy is reduced by 40–50% [15]. In addition, there is a decrease in the procoagulant potential, which indicates a correctly selected dose of the IA and high efficiency of treatment. The accumulation of functionally inactive forms of prothrombin in the blood plasma of patients of the first group also confirms a significant increase in the clotting time of blood plasma in the APTT screening test.

Comparative analysis of the results obtained during the activation of prothrombin in the blood plasma of patients with thromboplastin and ecamulin allowed us to identify a group of patients with low sensitivity to IA drugs (group 2). This conclusion was made on the basis that the level of prothrombin determined using thromboplastin and ecamulin was the same, which indicates the absence of decarboxylated forms of prothrombin. In such patients, either the dose of IAs should be increased to control the occurrence of decarboxylated prothrombin, or the IAs should be discontinued in favor of other anticoagulants.

For patients of the third group, there is a risk of bleeding: the content of total prothrombin is <70%, and the level of functionally active prothrombin is reduced to 20–30%. The reason for the decrease in the level of the latter may be the high sensitivity to the IAs. In these patients, IAs are contraindicated [18]. Perhaps, in such cases, the dose of IAs should be reduced and additional monitoring is needed to determine the reasons for the decrease in the total level of prothrombin.

It should also be noted that different sensitivity of patients to IAs, in particular to warfarin, can be genetically determined. Therefore, an individual approach to the dosage of IA based on the results of genetic testing can contribute to reducing the risk of hemorrhagic complications [13, 19].

Although the determination of prothrombin time is a generally accepted method for monitoring the IA effects, it alone is not enough. From a practical point of view, we consider it necessary to simultaneously perform the "ecamulin time" test, which allows us to determine the presence of functionally inactive (decarboxylated) forms of prothrombin. The need to control the content of decarboxylated forms of prothrombin is due to the individual sensitivity of patients to IAs.

Using an individualized test-based approach to IA dosage may help reduce the risk of hemorrhagic complications.

This work was carried out in the framework of the basic theme of the Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine № 0112U002624 "Mechanisms of regulation of intracellular signaling networks, intercellular and intermolecular interactions" (2012–2016).

REFERENCES

1. *Grober U., Reichrath J., Holick M. F., Kisters K.* Vitamin K: an old vitamin in a new perspective Dermatoendocrinol. 2015, 6(1), e968490. https://doi.org/10.4161/19381972.2014.968490
2. *Zirlik A., Bode C.* Vitamin K antagonists: relative strengths and weaknesses vs. direct oral anticoagulants for stroke prevention in patients with atrial fibrillation. *J Thromb Thrombolysis.* 2017, 43(3), 365–379. https://doi.org/10.1007/s11239-016-1446-0.
3. *Alquwaizani M., Buckley L., Adams C., Fanikos J.* Anticoagulants: A Review of the Pharmacology, Dosing, and Complications. *Curr Emerg Hosp Med Rep.* 2013, 1(2), 83–97. https://doi.org/10.1007/s40138-013-0014-6.
4. *Oscar M. P. Jolobe.* A comparison between vitamin K antagonists and new oral anticoagulants. *Br J Clin Pharmacol.* 2017, 83(11), 2589–2590. https://doi.org/10.1111/bcp.13347.
5. *Lipatova N. A., Titaeva E. V., Dobrovolskii A. B.* Vitamin K antagonists: mechanism of anticoagulant action and laboratory control of therapy. *Klin. Lab. Diagnostika.* 2006, 5, 25–33 (In Ukrainian).
6. *Brenner B., Kuperman A., Watzka M., Oldenburg J.* Vitamin K-dependent coagulation factors deficiency. *Semin. Thromb. Hemost.* 2009, 35(4), 439-46. https://doi.org/10.1055/s-0029-1225766.
7. *Margueritta S. El Asmar, Naoum J. J., Arbid E. J.* Vitamin K Dependent Proteins and the Role of Vitamin K2 in the Modulation of Vascular Calcification: A Review. *Oman Medical Journal.* 2014, 29(3), 172—77. https://doi.org/10.5001/omj.2014.44.
8. *Dolgov V. V., Svirin P. V.* Laboratory diagnostics of hemostasis disorders. M.-*Tver:* OOO "*Izdatelstvo "Triada""*, 2005. 227 s (In Russian).
9. *Solovjov D. A, Platonova T. N., Ugarova T. P.* Purification and characterization of ecamulin — a new prothrombin activator from the Echis multisquamatus snake venom. *Biochemistry (Mosc).* 1996, 61(6), 785–93.
10. *Castellone D. D., Van Cott E. M.* Laboratory monitoring of new anticoagulants. *Am. J. Hematol.* 2010, 85, 185–87. https://doi.org/10.1002/ajh.21607.
11. *Geoffrey Kershaw, Emmanuel J. Favaloro.* Laboratory identification of factor inhibitors: an update. *Pathology.* 2012, 44(4), 293–302. https://doi.org/10.1097/PAT.0b013e328353254d.
12. *Korolova D., Chernyshenko V., Platonova T., Chernyshenko T., Lugovskoy E.* Detection of prethrombin 1 in human blood plasma. *IBRR*, 2016, 5(2), 1–7. https://doi.org/10.9734/IBRR/2016/24683
13. *Danciger J.* Vitamin K-dependent Proteins, Warfarin, and Vascular Calcification. Clin. *J. Am. Soc. Nephrol.* 2008, 3, 1504–10. https://doi.org/10.2215/CJN.14180920.
14. *Samuelson B. T., Cuker A.* Measurement and reversal of the direct oral anticoagulants. *Blood Rev.* 2017, 31(1), 77–84. https://doi.org/10.1016/j.blre.2019.100593.
15. *Kini R. M.* The intriguing world of prothrombin activators from snake venom. *Toxicon.* 2005, 45, 1133–1141. https://doi.org/10.1016/j.toxicon.2005.02.019.

16. *Korolova D. S., Vinogradova R. P., Chernyshenko T. M., Platonova T. M., Volkov G. L.* The use of ekamulin from the poison of the polychaete epha in clinical laboratory diagnostics. *Lab. diagnostika*. 2006, 37(3), 18–22. (In Ukrainian).
17. *Korolova D. S., Deev V. A., Kypovska S. I., Chernyshenko T. M., Platonova T. M., Lugovskoj E. V.* Determination of functionally inactive forms of prothrombin to control the effectiveness of treatment with indirect anticoagulants. *Lab. diagnostika*. 2009, 2(48), 3–12.
18. *Lori-Ann Linkins.* Bleeding risks associated with vitamin K antagonists. *Blood Reviews*. 2013, 27, 111–118. https://doi.org/10.1016/j.blre.2013.02.004.
19. *Haug K.B.F., Sharikabad M. N., Kringen M. Sigrid Narum, Stine T. Sjaatil, Per Wiik Johansen, Peter Kierulf, Ingebjørg Seljeflot, Harald Arnesen, Odd Brørs.* Warfarin dose and INR related to genotype of CYP2C9 and VKORCI in patients with miocardial infarction. *Thromb. J.* 2008, 6(7), 557–62. https://doi.org/10.1186/1477-9560-6-7

ОПТИМІЗАЦІЯ МЕТОДУ ОЦІНЮВАННЯ ЕФЕКТИВНОСТІ ТЕРАПІЇ З ВИКОРИСТАННЯМ АНТИКОАГУЛЯНТІВ НЕПРЯМОЇ ДІЇ

Д. С. Корольова[1], *О. В. Горницька*[1], *В. А. Дєєв*[2], *В. І. Гришук*[1], *Т. М. Чернишенко*[1], *Т. Н. Платонова*[1], *В. О. Чернишенко*[1]

[1]Інститут біохімії ім. О.В. Палладіна НАН України, Київ
[2]Національний інститут хірургії та трансплантології ім. О. О. Шалімова Національної академії медичних наук України, Київ

E-mail: platonovatn@gmail.com

Лікування непрямими антикоагулянтами (антагоністами вітаміну К) потребує індивідуального підходу для контролю загального рівня протромбіну та накопичення його декарбоксильованих форм.

Мета. Оптимізувати метод моніторингу терапії непрямими антикоагулянтами.

Методи. Проведено аналіз 41 зразка плазми крові пацієнтів із серцево-судинною патологією. Використано методи лабораторної діагностики для визначення активованого часткового тромбопластинового часу (АЧТЧ), протромбінового часу (ПЧ), екамулінового часу (ЕЧ), статистичний аналіз даних («Statistica 7»).

Результати. Тест АЧТЧ дав змогу виявити індивідуальну чутливість пацієнтів до непрямих антикоагулянтів. Зокрема, у 20% пацієнтів виявлено зниження загального рівня протромбіну, що разом з накопиченням його декарбоксильованих форм призводить до розвитку кровотечі. Індивідуальну нечутливість до дії антагоністів вітаміну К було визначено в 11% пацієнтів.

Висновок. Для контролю ефективності терапії непрямими антикоагулянтами нами розроблено тест, в якому як активатор протромбіну використано екамулін (протеаза з отрути *Echis multisquamatis*), який може активувати не тільки функціонально активний протромбін, але і його декарбоксильовані форми. Застосування екамуліну одночасно з тромбопластином дає змогу визначати в плазмі крові вміст не тільки функціонально активного протромбіну, але й загального рівня протромбіну, що уможливлює контроль накопичення декарбоксильованого протромбіну.

Ключові слова: протромбін, вітамін К, непрямі антикоагулянти, тромболітична терапія.