METHOD TO ASSESS THE EFFECTS OF BIOACTIVE COMPOUNDS SOLUTIONS ON BLOOD CLOTTING

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The search for new anticoagulants requires simple and affordable methods for primary determination of their activity. Clotting tests are widely used for laboratory evaluation of the hemostatic system. These are model studies that assess the state of the hemostatic system from a clinical point of view based on the fibrin clot formation time. Reagents and instruments for such tests are produced in Russia, they have low manufacturing cost and are easy to use. However, it is necessary to make a few modifications to the measurement methods to assess the anticoagulant activity. The study was aimed to demonstrate performance of the protocol for testing the solution anticoagulant activity using the modified standard clinical tests involving measurement of the activated partial thromboplastin time (aPTT), prothrombin time (PT), and thrombin time (TT). Reagents for measurement of aPTT, PT, and TT were used, along with the domestically produced heparin and two recombinant anticoagulant proteins from the medicinal leech obtained in our laboratory. Clotting tests were performed with the addition of anticoagulants to the reaction mixture were performed; performance and applicability limits of the methods used were determined. When studying hirudin, heparin, and cysteine-rich anticoagulant of medical leech using measurement of aPTT, TT, and PT, a dose-dependent increase in clotting time was demonstrated. The methods' compatibility with the use of various common components of buffer solutions used in biochemical tests was determined. It was shown that the slightly modified standard blood clotting tests for determination of hemostatic parameters could be used to test new potential anticoagulants.

Keywords: activated partial thromboplastin time, prothrombin time, thrombin time, anticoagulant, leech

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МЕТОДИКА ОЦЕНКИ ВЛИЯНИЯ РАСТВОРОВ БИОЛОГИЧЕСКИ АКТИВНЫХ ВЕЩЕСТВ НА КОАГУЛЯЦИЮ

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Поиск новых антикоагулянтов требует простых и недорогих методов первичного определения их активности. Для лабораторной оценки состояния системы гемостаза широко используют клоттинговые тесты, т. е. модельные исследования, оценивающие с клинической точки зрения состояние системы гемостаза по скорости образования фибринового сгустка. Реактивы и приборы для их выполнения производят в России, они имеют низкую себестоимость и просты в применении, однако для исследования активности антикоагулянтов в методики измерения необходимо внести некоторые изменения. Целью работы было показать работоспособность протокола тестирования антикоагуляционной активности растворов с помощью модифицированных стандартных клинических тестов на измерение активированного частичного тромбопластинового времени (АЧТВ), протромбинового времени (ПВ) и тромбинового времени (ТВ). Использовали реактивы для измерения АЧТВ, ТВ и ПВ, а также гепарин отечественного производства и два рекомбинантных белка-антикоагулянта медицинской пиявки, полученные в нашей лаборатории. Проводили клоттинговые тесты с добавлением в реакционную смесь антикоагулянтов, определяли работоспособность и границы применимости использованных методов. При исследовании гирудина, гепарина и цистеин-богатого антикоагулянта медицинской пиявки с помощью измерения АЧТВ, ТВ и ПВ продемонстрировано дозо-зависимое увеличение времени образования сгустков. Определена совместимость методов с использованием некоторых распространенных компонентов буферных растворов, используемых в биохимических исследованиях. Показано, что после небольших модификаций стандартных клоттинговых методов определения параметров гемостаза можно использовать их для тестирования растворов новых потенциальных антикоагулянтов.

Ключевые слова: активированное частичное тромбопластиновое время, протромбиновое время, тромбиновое время, антикоагулянт, пиявка

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Simple, cheap, and well mastered blood clotting tests (activated partial thromboplastin time (aPTT), prothrombin time (PT), and thrombin time (TT)) are used in clinical practice [1–3]. These tests are developed to determine homeostasis effectiveness in blood plasma of patients. aPTT allows one to estimate functioning of the intrinsic coagulation activation pathway, PT measurement is used to determine coagulation in case of extrinsic pathway activation, while TT demonstrates the effectiveness of thrombin-induced fibrinogen activation and fibrin polymerization. When conducting research focused on finding new anticoagulants, there is a need for rapid and affordable *in vitro* testing of the candidate substances. Furthermore, one has to deal

with various complex protein mixtures, extracts, and similar material. At the initial stage of research, simple and affordable primary screening methods are necessary.

The method to determine the effects of potential anticoagulant proteins derived from the medicinal leech (Hirudo medicinalis) has been developed and is used in our laboratory. We provide the measurement protocols used and demonstrate their effectiveness using three substances inhibiting blood clotting. The first substance, the cysteine-rich anticoagulant from the medicinal leech (CRA), is the recently explored [4] anticoagulant protein related to antistasin [5]. The second research object is the recombinant hirudin protein, a well-known anticoagulant from the medicinal leech [6]. The third anticoagulant tested is heparin, an oligosaccharide very commonly used in clinical practice [2, 7].

The study was aimed to demonstrate the efficacy of the testing protocol for assessing the anticoagulant activity of solutions using modified standard clinical tests involving measurement of aPTT, PT, and TT.

METHODS

Equipment

APG4-03-Ph hemostasis analyzer (EMCO; Russia). Disposable coagulometer cuvettes with balls (EMCO; Russia). Measurement was performed using the analyzer optical channel (Optics mode).

Reagents

PG-7/1 reagent kit for determination of aPTT in blood plasma by the clotting method (aPTT test) (RENAM; Russia).

Thrombin-Reagent PG-9A kit for determination of TT (RENAM; Russia).

MLT-Thromboplastin reagent for determination of PT (EMCO; Russia).

KM-1 control plasma (Plasma-N) (RENAM; Russia).

Anticoagulants

The cysteine-rich anticoagulant (CRA) from the medicinal leech [4] and recombinant hirudin (Hir) from the medicinal leech [4] obtained in our laboratory, as well as the solution of medium molecular weight unfractionated sodium heparin (Hep) in the industrially produced ampoules (Synthesis; Russia) were used as anticoagulants. Serial dilutions of anticoagulants were prepared for the study. Anticoagulants were diluted with the 10 mM TrisCl solution, pH 7.5.

Blood clotting tests

When working with anticoagulants, each sample was measured in four replicates in parallel using four coagulometer measurement cells. The mean and standard deviation were calculated for the values obtained. Curves of clot formation time versus anticoagulant concentration were plotted based on the data acquired (see Figure). To determine the effects of various solutions on the measurement results, each sample was measured in two replicates in parallel using two coagulometer measurement cells of one pair. The mean was calculated for the values obtained and entered in the table (see Table).

Activated partial thromboplastin time

The 2X aPTT reagent was prepared for aPTT measurement. For that 2 mL of water were added to the vial with the lyophilized aPTT reagent, which constituted half of the volume recommended for clinical aPTT test. One milliliter of water was added to the vial with the lyophilized control blood plasma, as recommended by the manufacturer. The vials were kept for 30 min at room temperature with occasional stirring until the precipitate was completely dissolved. The calcium chloride solution was poured in the temperature-controlled coagulometer cell to be heated to 37 °C. A total of 50 µL of control plasma, 25 µL of the test solution, and 25 µL of the 2X aPTT reagent were added to the coagulometer cuvette, then the contents of the cuvette was mixed by triple pipetting. A magnetic ball was put in the cuvette; the cuvette was placed in the temperature-controlled timer coagulometer cell for 3 min. After the incubation was over, cuvettes were transferred to the measurement coagulometer cells; 50 µL of the heated calcium chloride were added in the Autostart mode. Clot formation time was recorded.

Thrombin time

The thrombin reagent with the concentration of 6 U/mL was used to determine TT. To prepare the reagent, 2.7 mL of water and 0.3 mL of the concentrated solvent (buffer solution from the kit) were added to the vial containing the lyophilized thrombin. One milliliter of water was added to the vial with the lyophilized control blood plasma, as recommended by the manufacturer. The vials were kept for 30 min at room temperature with occasional stirring until the precipitate was completely dissolved. The thrombin reagent solution was poured in the temperature-controlled coagulometer cell to be heated to 37 °C. A total of 100 µL of control plasma and 50 µL of the test solution were added to the coagulometer cuvette; the contents of the cuvette was mixed by triple pipetting. After that a magnetic ball was put in the cuvette; the cuvette was placed in the temperature-controlled timer coagulometer cell for 3 min. After the incubation was over, cuvettes were transferred to the measurement coagulometer cells; 50 µL of the heated thrombin reagent were added in the Autostart mode. Clot formation time was recorded.

Prothrombin time

The 2X thromboplastin solution was used to determine PT. To prepare the solution, 3 mL of water were added to the vial containing the lyophilized thromboplastin-calcium mixture, which constituted half of the volume recommended for clinical PT test. One milliliter of water was added to the vial with the lyophilized control blood plasma, as recommended by the manufacturer. The vials were kept for 30 min at room temperature with occasional stirring until the precipitate was completely dissolved. The thromboplastin solution as poured in the temperature-controlled coagulometer cell to be heated to 37 °C. A total of 50 µL of control plasma and 50 µL of the test solution were added to the coagulometer cuvette; the contents of the cuvette was mixed by triple pipetting. After that a magnetic ball was put in the cuvette; the cuvette was placed in the temperature-controlled timer coagulometer cell for 3 min. After the incubation was over, cuvettes were transferred to the measurement coagulometer cells; 50 µL of the heated thromboplastin solution were added in the Autostart mode. Clot formation time was recorded.

Statistical analysis

The nonparametric Mann–Whitney U-test and Python (v. 3.12) (Python Software Foundation; USA) were used for statistical

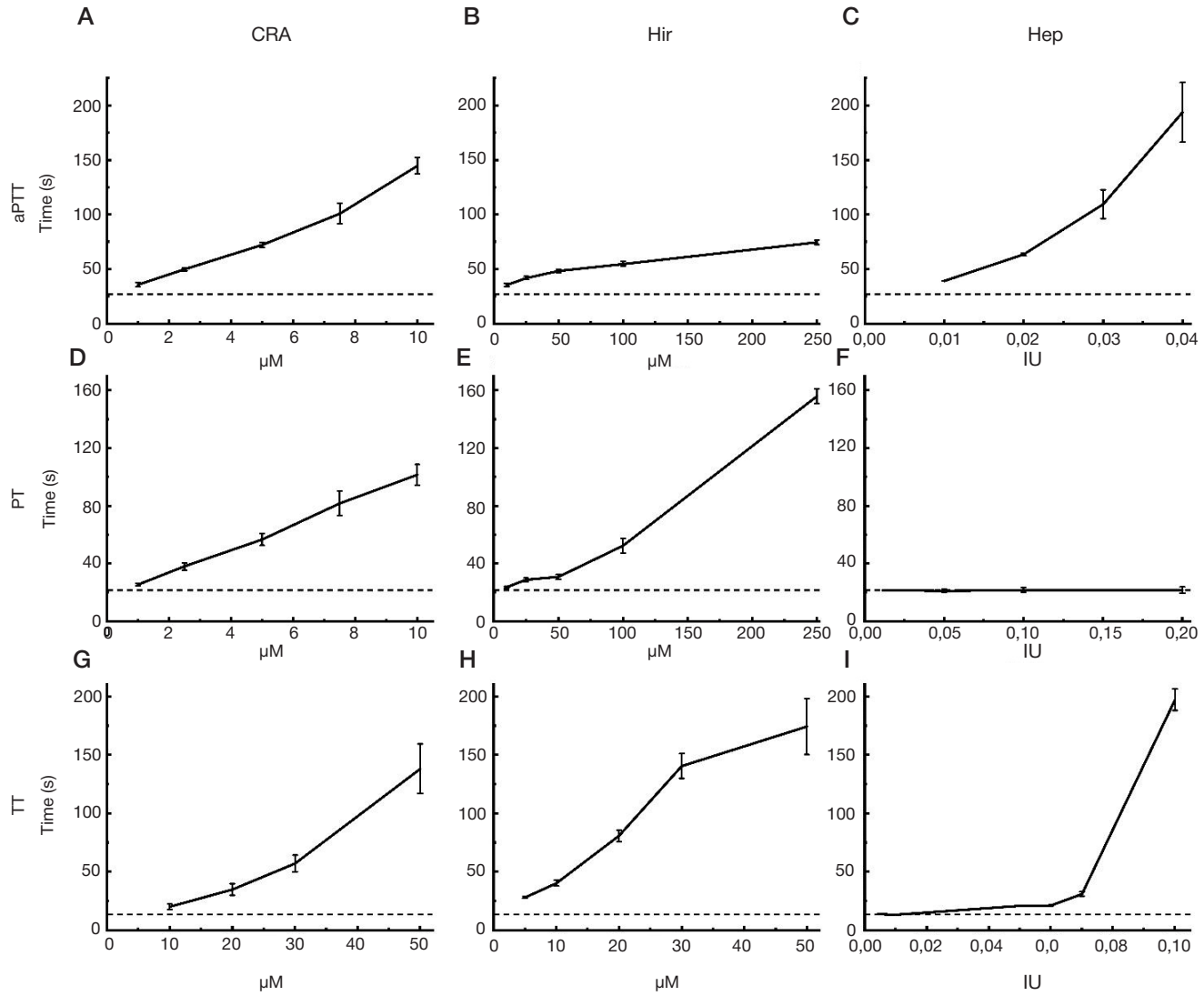

Fig. 1. Results for aPTT, PT, and TT measurements for the solutions of three anticoagulants: cysteine-rich anticoagulant (CRA) derived from the medicinal leech, hirudin (Hir), and heparin (Hep). Concentrations of CRA and Hir are measured in μM of protein in the test solution; the total amount of IU per reaction is provided for Hep. The mean clot formation time for the control sample (10 mM TrisCl, pH = 7.5) is shown as the *dashed line*. The data are provided as the mean values of clot formation time and standard deviations. Four independent measurements per point were performed

analysis when comparing the samples containing test proteins and control plasma.

RESULTS

The aPTT determination results are provided in Fig. 1 A–C. A clear increase in the clot formation time with increasing active substance concentration is reported for all three anticoagulants.

The PT measurement results are provided in Fig. 1D–F. As in case of aPTT measurement, the increase in the reaction mixture coagulation time with increasing concentration is reported for CRA (Fig. 1D) and Hep (Fig. 1E). Heparin that has shown high activity in the aPTT test exerts no activity during TT measurement (Fig. 1F).

The TT measurement results are provided in Fig. 1G–I. All three anticoagulants show a dose-dependent increase in the clot formation time.

To determine compatibility of the reported methods with the common buffer solutions used in biochemical analysis, we examined their effects on the clot formation time for all three tests reported. The results are summarized in the Table. In the majority of cases no effect or negligible effect was observed. However, 1% β-mercaptoethanol prevented coagulation during aPTT and PT measurement, while the 1M NaCl solution caused a significant increase in the values in all three tests.

DISCUSSION

Boundary applicability conditions are the key parameters for any method. When conducting the experiments, in addition to automatic recording of clot formation time by the device, we performed visual monitoring of the reaction mixture state. When the anticoagulant concentrations in the measurement cuvettes were high, apparent coagulation irregularity, individual clumps and harnesses were observed around the magnetic balls, along with the mobility of balls after the coagulometer sensor activation. In this context the results cannot be considered reliable, so the upper measurement threshold should be limited to a hundred of seconds. Thus, in all three tests the anticoagulant concentrations should be selected so that the clot formation time is about 100 s at the maximum test concentration. If clot formation does not occur within this time during measurement, the sample measured should be further diluted. It should also be noted that reagents and control plasma from different production batches yield slightly different results during measurement of aPTT, TT, and PT. Therefore, it is

Table. Effects of various solutions on the aPTT, TT, and PT determination

Sample/Test	aPTT	TT	PT
Water (K)	28.7 ± 1.0	12.8 ± 0.6	15.9 ± 0.5
PBS[1]	29.7 ± 0.6	18.0 ± 0.4	18.6 ± 0.1
Tris-HCl 100 mM	28.0 ± 1.0	17.4 ± 0.4	17.3 ± 0.3
NaCl 9 g/L	29.1 ± 0.4	17.3 ± 0.3	19.0 ± 1.0
NaCl 1 M	62.0 ± 2.0	114.0 ± 18.0	136.8 ± 0.5
SDS 0.1%	31.0 ± 2.0	13.1 ± 0.7	16.1 ± 0.1
SDS 0.01%	28.5 ± 0.2	12.1 ± 0.8	15.2 ± 0.1
1 M urea	29.0 ± 1.0	33.7 ± 0.1	21.3 ± 0.4
0.1 M urea	28.1 ± 0.7	13.9 ± 0.4	15.7 ± 0.1
β-mercaptoethanol 1%	n. d.[2]	16.0 ± 2.0	n. d.[2]
β-mercaptoethanol 0.1%	33.0 ± 2.0	13.0 ± 2.0	21.8 ± 0.6
Triton X-100 1%	26.0 ± 1.0	13.0 ± 0.1	n. d.[2]
DTT 1 mM	31.0 ± 2.0	14.0 ± 0.4	19.4 ± 0.5

Note: mean clot formation time in seconds ± standard deviation is provided; [1] — phosphate buffer solution (20 mM sodium phosphate, 8 g/L sodium chloride, pH = 7.4); [2] — no clot was formed, no value is available.

strictly necessary to perform all the series of measurements of each sample (or several samples to be compared) and control samples using reagents from the same batch.

Various normalization methods can be used to compare the results obtained at different times using different reagents. For example, the international normalized ratio (INR) is widely used in clinical diagnosis for PT [1–3]. It seems possible to use the relative value (C_S), calculated as a quotient of the clot formation time of the test sample (t_S) to the clot formation time of the control sample (tC), for screening tests: $CS = t_S/t_C$.

When they suggest to perform three different tests per test sample, the authors assume that the search for substances with unknown characteristics and mechanism of action, the only essential feature of which is the ability to inhibit coagulation, will be conducted. In this regard we believe that it is necessary to use all three tests at once. In such situation the researchers may prefer this or that test, for their own reasons. The differences in the effects of three anticoagulants used in this study are pretty obvious.

An obvious dose-dependent effect is reported for all three test substances in the aPTT test. However, CRA shows the time (Fig. 1A) comparable with that of Hir (Fig. 1B) at the molar concentrations smaller by an order of magnitude. For example, the clot formation time of about 50 s is achieved at the CRA concentration of 2.5 μM in the sample, as for hirudin — only at the concentration of 50 μM. It can be concluded that CRA significantly more effectively inhibits activation of intrinsic blood clotting pathway compared to Hir. Predictably, Hep also effectively inhibits coagulation (Fig. 1C). However, it is impossible to directly compare its specific activity with the activity of the studied proteins, since the concentration of the Hep dosage form is specified in international units (IU) of activity only.

A slightly different pattern is observed for PT measurement (Fig. 1D–F). As for CRA (Fig. 1D) and Hep (Fig. 1E), the increase in the reaction mixture coagulation time with increasing concentration is observed, the same as in the aPTT test. At the same time, heparin that has shown high activity in the aPTT test causes no increase in the clot formation time during TT measurement (Fig. 1F). This is due to the fact that it inhibits factors Xa and IIa, as well as factors of the intrinsic activation pathway, not directly, but with mediation from antithrombin [7].

When determining TT (Fig. 1G–I), remarkable is that in this case there is a situation opposite to measuring aPTT: Hir (Fig. 1H) shows higher activity, than CRA (Fig. 1G). This may be due to the fact that Hir is a specific inhibitor of thrombin (factor IIa) [6], while CRA inhibits not only thrombin, but also other proteinases of blood clotting cascade [4]. As a result, the CRA activity is more obvious during measurement of aPTT due to cumulative effect. In this case the measurement range of Hep (Fig. 1I) is severely narrowed. When 0.07 IU of Hep are added to the reaction, the coagulation time is 29.3 ± 2.5 s; no full-fledged clot is formed, when 0.1 IU are added to the reaction.

When performing primary search for the substances preventing blood clotting, the researchers often have limited knowledge about their nature and mechanism of action. It is necessary to first detect the active substance and obtain its relatively pure form to thoroughly investigate these aspects. This problem is particularly evident during the study of the complex mixtures of natural origin, such as saliva of blood-sucking animals, secretions, extracts, etc. The researchers can observe manifestations of biological activity, but at this stage do not understand the underlying mechanisms. That is why we think it is reasonable to use three different tests targeting three different parts of blood clotting cascade (intrinsic pathway (aPTT), extrinsic pathway (PT), and terminal phase (TT)) at once. Using three anticoagulants of different nature and properties we have shown that these behave differently in these tests.

During testing the new potential anticoagulants can exist in the form of solutions containing various low molecular weight substances. For example, when studying recombinant anticoagulant proteins, these turn out to be dissolved in buffer solutions, the composition of which depends on the specific extraction method, after the chromatographic purification or refolding. In each specific case, it is strictly necessary to perform control measurements with the buffer solution that is identical to the solution, in which the test substance is dissolved. However, some solutions can be incompatible with the measurement methods used. We tested the effects of some commonly used components of buffer solution used in

biochemical studies on the aPTT, TT, and PT measurement. The results are provided in the Table. In the majority of cases, the impact of the studied solution on the test results was negligible. However, it is impossible to use the solutions with high ionic strength (1M NaCl) in all tests, while strong reducing agents (β-mercaptoethanol) cannot be used when determining aPTT and PT. At the same time, the solutions containing weaker reducing agents (DTT), moderate amounts of detergents (Triton X-100, sodium dodecyl sulfate (SDS)) or 0.1 M urea can be used for measurement.

CONCLUSIONS

The paper provides modifications of methods to determine aPTT, PT, and TT using the common reagents and the domestically produced coagulometer. Modifications include changes in the reagent volume and incubation time and require no additional reagents or equipment for measurement. The methods described can be useful when performing the search for new anticoagulants and studying the effects of various substances on blood clotting.

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