Quantification of coagulation factors and inhibitors
Still a special task

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Summary
This is a very short review on quantitative coagulation factor assays for the beginner. For systematic training several excellent textbooks in German language are available. Quantitative functional assays of coagulation factors and of physiological inhibitor proteins are based on the principle of parallel-line or slope ratio bioassays. With the modern analyzers the test procedure follows the example of clinical chemistry: a single test plasma dilution read from an actual calibration curve, regular internal and external quality control. If there are unexpected results or a suspicion of hae-mophilia we recommend to repeat the assay with three different predilutions of the test plasma. The resulting potency estimates should not deviate by more than 10-15% from their average. Otherwise the assay is invalid and requires further investigation (e.g. search for inhibitors). Special problems may complicate diagnostic activities. As an example discrepancies between factor VIII one-stage clotting and chromogenic assays are discussed.

SLeschlüsselwörter
Quantitative Gerinnungsfaktorenbestimmung

Zusammenfassung

Messung von Gerinnungsfaktoren und Inhibitoren: Immer noch eine anspruchsvolle Aufgabe
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An educational review of quantitative assays of coagulation factors and coagulation inhibitors on a few pages may only address some aspects of relevance from the viewpoint of the author. However I am confident that laboratory people interested in haemostasis tests will easily find excellent sources of information in German. Walking through our department of Laboratory Medicine I found standard textbooks of clinical chemistry and laboratory medicine (8, 18) containing large sections on haemostasis covering assay principles, diagnostic steps, pathophysiology and clinical information. The authors of these chapters are outstanding and experienced haemostasis specialists and excellent teachers. As an advantage for the student of laboratory science these textbooks present in-depth information on various assay techniques and concepts of quality management common to the laboratory disciplines of clinical chemistry, haematology, molecular medicine, microbiology and haemostasis. There are handy books of this kind that are easily carried to the bench and any other place (4). Comprehensive information on all aspects of haemostaseology will be found in a well known pocket book (2) and in several monographies by other hospital or industry based authors.

The topics I would like to touch specifically relate to assay principles, performance and some special problems.

Preanalytical conditions

For coagulation assays preanalytical conditions are very important:
● treatment with drugs acting on haemostasis (heparin, hirudin, thrombin inhibitors etc.),
● quality of blood sampling and
● rapid transportation to the laboratory.

Mysterious results may induce laborious detective activities to find appropriate explanations.

Optimal blood sampling by venepuncture is often difficult, especially in patients of intensive care units. Blood obtained from venous access devices (intravenous catheters, shunts for haemofiltration etc.) may be “contaminated” by infusion fluids or heparin (sometimes only used for patency of the access). If no heparin is present at least 10 ml blood should first be discarded before an appropriate sample for coagulation assays can be obtained. In the presence of heparin even extensive flushing of the system with saline followed by the removal of 20-30 ml blood may not solve the problem. We recommend to measure the thrombin time in any plasma sample sent for clotting tests of a hospitalized patient as heparin is virtually “ omnipresent” in hospitals. If no blood sample free of heparin can be obtained plasma may be treated with heparinase before assaying heparin-sensitive variables.
For tests after substitution of clotting factor concentrates blood sampling should be done on the arm opposite to the factor administration. The same holds true for tests of heparin activity (aPTT, thrombin time, anti-Xa activity).

Considering the interval between blood sampling and assay performance (or sample refrigeration below -20°C) recommendations may vary depending on the test required. Factor VIII and factor V are rather labile ex vivo. In our laboratory we make the compromise to accept citrated blood samples within four hours after venepuncture for clotting factor tests and within two hours for heparin activity.

Principles of quantitative coagulation assays

Still the most typical assays of the haemostasis laboratory are quantitative coagulation assays. However the assay spectrum has much changed within 25 years. Instead of handling platinum hooks, glass tubes and pipettes at the water bath technicians control the action of autoanalyzers, ELISA readers, thermocyclers and platelet aggregometers. In addition there may be POCT devices for whole blood analysis. Another specialty are chromogenic substrate assays mainly used for inhibitors (antithrombin, protein C, antiplasmin etc.). These tests have recently been reviewed in “Hämostaseologie” by Rosen (17).

Bioassay principle

Quantitative coagulation factor assays are typical bioassays. They use a complex biological system (coagulation activation and clot formation), compare a test material (patient plasma) with a standard preparation (calibration plasma). The biological response are clotting times of selected dilutions of patient and standard plasma. The biological systems used are the intrinsic clotting system for factors XII, XI, IX, VIII and the extrinsic clotting system for factors VII, X, V, and II. Plasmas deficient or selectively depleted of the clotting factor to be measured are mixed with dilutions (e.g. 1:5, 1:10, 1:20, 1:40, 1:80) of the test or the standard plasma and then activated with aPTT reagent + Ca²⁺ and thromboplastin + Ca²⁺, respectively. Usually the 1:5 or 1:10 dilutions of test and standard plasma are assigned 100% activity of the respective material. The quantitative evaluation of the potency of the test sample relative to the standard is based on the plot of clotting times versus percent activity of the corresponding plasma dilution. The plot of clotting times or their logarithm (y-axis) versus logarithm of relative percent activity (x-axis) can be fitted to straight lines. The lines of the test plasma and standard plasma dilutions should be parallel. The assay is therefore called parallel-line assay. The horizontal distance between the parallel lines reflects the relative potency of the test plasma versus the standard plasma. Absence of parallelism shows that the quantitative assay is invalid. Reasons for non-parallelism could be inhibitors (e.g. antibodies against clotting factors, heparin, hirudin etc.) in the test plasma. Theory and mathematics of parallel-line assays are described in the classical textbook by Finney (7) and their applications to clotting tests in a textbook article by Barrowcliffe et al. (1) and elsewhere. Parallel-line assay calculations require replicate measurements of 3-4 different dilutions of test and standard plasmas. There are computer programmes for statistical calculations (5, 10, 19).

Another form of bioassay is the slope ratio assay. The biological response (y-axis) is plotted against the relative activities of the corresponding dilutions on the x-axis (linear). The plots of each dilution curve should fit a straight line. As a necessary criteria of assay validity the two lines should intersect on the y-axis. The ratio of the slopes of the two straight lines gives the relative potency of the test plasma versus the standard plasma. In principle the slope ratio assay can also be used for quantitative clotting assays, as the diluent value (0%) also results in a finite clotting time. The slope ratio assay fits quite well to chromogenic substrate assays.

It should be mentioned that any suitable mathematical transformation of the response variable on the y-axis is allowed in order to obtain straight lines for both types of assays.

Autoanalyzers for coagulation tests

Well performed bioassays are quite demanding with respect to manpower, time and reagent costs. Their advantage consists in the actual information about validity and precision of the test system. The clinical haemostasis laboratory will usually not have the resources to do parallel line assays for the large number of tests required every day. The introduction of precise autoanalyzers for coagulation tests may compensate for this deviation from the theoretical optimum. In order to prove the assay validity three different dilutions of a selected sample may be assayed. After correction for the dilution factors the resulting potencies should be equal (deviation from their average not more than 10-15%). This test rule has universal validity for any type of assay. A prerequisite is the use of the appropriate diluent.

Today reagents for coagulation factor assays are commercially available. As for clinical chemistry companies may offer autoanalyzers and tests adapted to these machines with their own reagents. They provide factor deficient plasma, activators (aPTT reagents, thromboplastin), calibration and control material. There is teaching and training for the laboratory technicians.

Common technical standards — DIN standards

The haemostasis specialist might be afraid of losing control about his laboratory. However, there are common rules, the DIN standards, accepted by manufacturers. The DIN standards are formulated by the Technical Committee C5 “Hämostaseologie” of Section C “Laboratory medicine and clinical chemistry” of the Medical Standards Committee of DIN (Arbeitsausschuss C5 “Hämostaseologie”, Fachbereich C “Laboratoriumsmedizin und Klinische Chemie”, Normenausschuss Medizin DIN). 22 DIN standards have been published in 2002 by Prof. H. Beeser, chairman for many years of the Technical Committee C5 (3). The committee is comprised of physicians and scientists from universities, research institutes and diagnosticians industry who are experi-
enced in the haemostasis field. I would like to recommend this DIN handbook 261 to each laboratory scientist and physician working in the haemostasis field. It contains definitions of important terms regularly used such as

- reference curve (Bezugskurve),
- calibration plasma (Kalibrierplasma),
- reference normal plasma (Referenznormalplasma),
- deficient plasma (Mangelplasma),
- one-stage-method (Einstufenmethode)

etc. It describes the structure of the assays that may then be adapted to different clotting analyzers.

**Quality control**

As in clinical chemistry precision and accuracy have to be secured by quality control samples with assigned values in the normal and in the pathological range. Important is the regular participation in external quality assessment programmes. By consensus the mean value of all participants is accepted as the reference or “true” value of the variable to be measured in the sample provided by the quality control centre. It is worthwhile to study the statistical reports carefully. Mean activities or concentrations and coefficients of variation may depend on the reagents used. For clotting assays coefficients of variation between laboratories tend to be higher for the factors of the intrinsic system (average about 15-30%) than for those of the extrinsic systems (about 10-20%). A recent paper (14) has looked at long-term results of laboratories participating in an external quality assessment programme for antithrombin, protein C and protein S. The coefficients of variation between laboratories have been lowest for antithrombin (about 7% for variables within the normal range, 9% for variables below the lower limit of reference interval) and highest for Protein S activity (about 15% and 35% respectively). The analysis allowed to estimate the long-term within-laboratory analytical coefficient of variation (LCVa). This gives an impression of the long-term analytical performance of the laboratory. The LCVa was lowest for antithrombin activity (7.6%) and highest for protein S activity (17.2%). Obviously there is an influence of analyte and method (antithrombin: chromogenic; protein S activity: clotting). However, the performance range of individual laboratories is broad. The authors conclude that variation of results between laboratories might be reduced if they improved their within-laboratory test performance.

**Special problems**

For several coagulation factors chromogenic substrate assays have been developed. Laboratories have mainly been interested in chromogenic assays for factor VIII in order to follow its plasma activity during substitution of haemophilia A patients with recombinant factor VIII (rFVIII). It has been known for several years that one-stage clotting assays tend to measure lower VIII:C activities after administration of rFVIII than chromogenic substrate assays. The difference may be 10-20% for full length rFVIII and even 40-50% for B-domain-deleted rFVIII. The problem may be attenuated for the one-stage clotting assay if a rFVIII plasma standard is used for calibration (12, 15). The problem has been critically reviewed by P. Lollard (13).

It appears that most laboratories still use one-stage clotting assays for most diagnostic purposes. One reason may be divergent VIII:C activities measured in mild (FVIII:C 5-40%) and moderate haemophiliacs (FVIII:C 1-5%). In our experience the chromogenic assay often assigns these cases to severe haemophilia A (FVIII:C <1%) despite using an extra calibration curve for values below 15%. On the other hand there are rare factor VIII missense mutations with higher FVIII:C activities measured by one-stage clotting assays than with chromogenic assays or with the classical two-stage clotting method. In these cases the chromogenic assay better reflects the factor VIII deficiency and the clinical severity. The reason may be the more rapid dissociation of the A2 unit of the activated factor VIII due to the Arg 531 His substitution (16).

One option for the laboratory is to have both assay methods available and to use them selectively. There are several other examples demonstrating the complexity of haemostasis diagnostics. We have already alluded to protein S assays above (6, 11).

**Conclusion**

Good results of internal and external quality assessment and knowledge of the peculiarities of the assays and of the clinical context are important prerequisites for good counselling by the academic person on duty for the haemostasis laboratory.

**References**


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