

Critical aspects in routine coagulation testing

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Abstract

The major improvements obtained in the quality of commercial reagents and in the accuracy of coagulometers has rendered comparability of the results among different laboratories a major task of the bodies devoted to standardization of coagulation testing. Commutability of results is of major importance especially in the monitoring of anticoagulant therapy. Critical aspects relate to the preanalytical phase, the analytical phase and to the expression of results with the adoption of proper correction factors - like the international sensitivity index (ISI) for thromboplastin reagents. Problems with the APTT in the monitoring of heparin treatment include the variable sensitivity of commercially available APTT reagents to clinically insignificant deficiencies of factors involved in the contact phase of coagulation and to the presence of lupus anticoagulants, which - in addition to the different sensitivity of the reagents to the anticoagulant effect of heparin - render commutability of results unworkable in practice.

The tasks of the routine coagulation laboratory should include the following: a) the identification of patients with congenital or acquired defects potentially leading to bleeding; b) the monitoring of anticoagulant therapy; c) the monitoring of the course of disseminated intravascular coagulation (DIC). The assays required to these purposes comprehend the prothrombin time (PT), the activated partial thromboplastin time (APTT), the thrombin time (TT), and the measurements of fibrinogen (Fib) and of fibrin specific degradation products (FbDP)(1).

At variance with most of the analyses of clinical chemistry the PT and the APTT do not measure a single analyte but are screening tests sensitive to single or multiple clotting factor deficiencies of the clotting cascade. These peculiar characteristic coupled with the low cost of the tests have suggested a wide range of applications. However, the very same properties are also responsible for the hard work required in the standardization of these assays. As pointed out in recent NCCLS documents (2,3), the PT and APTT tests have their original scope in the detection of clinically relevant coagulation defects, that is congenital deficiencies of the intrinsic or extrinsic coagulation pathways which may be responsible for a bleeding diathesis. The two tests are also commonly employed to detect acquired clotting factor deficiencies or inhibitors the most common of which is the broad spectrum of lupus anticoagulants. In screening for congenital clotting factor deficiencies, the characteristics of the reagents are of the utmost importance as they differ in the degree of sensitivity to moderate deficiencies of single factors. Ideally, neither test should be sensitive to a 50% deficiency in any single factor, because such a deficiency cannot be responsible for a clinically significant bleeding diathesis. Insensitivity to single factor deficiencies close to 50% also permits correction of clotting time prolongations in mixing experiments aimed to rule out the presence of inhibitors.

The PT and APTT tests are used in the large majority of laboratories to monitor oral anticoagulant and heparin treatments. The major improvements obtained in the quality of commercial reagents and in the accuracy of coagulometers has rendered comparability of the results among different laboratories a hard goal. Commutability of results is of major importance especially in the monitoring of oral anticoagulant therapy. Critical aspects relate to the preanalytical phase, the analytical phase and to the expression of results with the adoption of proper correction factors - like for instance the international sensitivity index (ISI) for thromboplastin reagents. Normal reference ranges should be obtained in each laboratory, keeping in mind lot-to-lot variations in the sensitivity of the same brand of reagent to factor deficiencies. Results should only be expressed as ratio of the clotting time of the test plasma to that of the normal plasma, to account for the wide differences in the clotting time of normal plasma with the different reagent method combinations (4,5).

A serious problem is represented by the definition and choice of the normal plasma required as the denominator in the expression of PT and APTT ratios. Uncertainty about the equivalence of fresh normal plasma samples - such as those utilized for the calculation of the mean normal prothrombin time (MNPT) - with lyophilized normal pooled plasma represents a cause of concern in the standardization of the prothrombin time (6). Requirement for stabilizers to avoid the loss of activity of all the relevant coagulation factors - and particularly of the labile factors V and VIII - and the effect of such stabilizers on the results of the prothrombin time has been extensively evaluated by manufacturers and investigators involved in quality assessment programs (7-11). There are definite advantages in the use of lyophilized plasmas. One major advantage of obtaining a reference normal plasma in the lyophilized form is to avoid collection and measurement of a relevant number of normal individual samples for the calculation of the MNPT (the denominator in the formula of the INR)(10). To the purpose of in-house calibration of lots of normal control plasma, the VDPGH (Verband der Deutschen Geräte-Hersteller) has prepared a lyophilized normal pooled plasma from 101 healthy blood donors (11). This plasma - named R82A - has been calibrated in a study involving manufacturing companies against fresh plasma pools using the reference thromboplastin BCT/099 and a large series of additional reagent/ method combinations (11). The results of the study indicated that the prothrombin time of this lyophilized plasma was 2% longer than that of fresh plasma pools with the combined thromboplastins and 6% longer with the plain thromboplastins. As no apparent effect of end-point detection methods did emerge, the authors concluded that the VDPGH reference plasma (R82A) could be used as a substitute for fresh normal pooled plasma with the use of different correction factors for plain and combined thromboplastins. Plasma R82A has also been evaluated against fresh individual normal plasmas (MNPT) using a combined reference thromboplastin (OBT/79) and a series of plain reference thromboplastins or candidate reference thromboplastins (12). In this study, there was no difference between the prothrombin time of the lyophilized plasma and the MNPT with the combined thromboplastin, but there was an excess 2% prothrombin time prolongation of the lyophilized plasma with plain thromboplastins. It was concluded that the VDPGH reference plasma could be used as a substitute for fresh normal plasma for calculation of the INR, but a correction should be made with plain thromboplastins. A IFCC collaborative study was started with the aims to prove the principle of calibration of plasma R82A against: a) the mean PT of at least 30 fresh individual normal plasmas and b) the mean PT of fresh plasma pools obtained from the same individuals using a variety of thromboplastins and end-point detection methods, and including in the evaluation the recently introduced recombinant thromboplastins along with the traditional plain and combined reagents. Participants in the study included laboratories from France, Germany, USA, Italy, Austria and the Netherlands. The results obtained confirm the data of the previous studies with respect to plain and combined thromboplastins but show a significant deviation from this behaviour of the recombinant thromboplastins, which record significantly longer clotting times in the lyophilized plasma than in fresh plasma (13). A major assumption for commutability of the results is that the reference and the routine method must have equal specificity for the analyte to be measured. Given the complexity of the analytes actually measured by the prothrombin time, it is surprising

that such small differences are detected with traditional plain and combined thromboplastins in fresh and in lyophilized normal plasma. The results obtained with the recombinant thromboplastins questions the concept of closely similar specificity of these reagents with extractive thromboplastins. Recombinant thromboplastins, due to their unlimited availability, safety from viral contamination and supposedly consistent reproducibility are being considered as future international reference thromboplastins. The data of the IFCC collaborative study cast a word of caution and call for additional experimentation before this is accomplished.

The APTT test primarily screens for intrinsic coagulation defects, is used for monitoring heparin anticoagulant therapy, detects other inhibitors of blood coagulation and monitors replacement therapies (factor assays). The APTT reagent-instrument combination should be able to detect abnormally prolonged results with plasmas that have less than 0.3 U/ml activity of any one of the following coagulation factors: VIII, IX, XI. However, a number of studies have shown considerable variation in the sensitivity of various APTT reagents to mild and moderate factor deficiencies (3). Also, a similar variable sensitivity of the APTT to circulating lupus anticoagulants has been reported. Likewise, marked variability in response to heparin - and lot-to-lot variability - has been reported with commercially available APTT reagents (3). We compared APTT results obtained with three reagent-instrument combinations (APTT STA-STA, Stago; APTT STA-ACL 300R, Instrumentation Laboratory, and Actin FS (Baxter)-MLA 1000) in normal controls and in patients on heparin therapy, with lupus anticoagulants and on stable oral anticoagulant treatment (Table 1). Clearcut differences were observed not only between reagents, but also with the same reagent depending on the instrument used.

TABLE 1. APTT values (Ratio, mean \pm SD) with the three reagent-method combinations in normal controls, patients on heparin treatment, patients with lupus anticoagulants (LA), and on oral anticoagulant treatment (OAT). Significance of the differences observed (*p* value) is also shown.

	Controls (n=45)	Heparin (n=56)	LA (n=7)	OAT (n=22)
Actin FS	.95 \pm .09	1.70 \pm .58	1.79 \pm .46	1.74 \pm .30
MLA 1000	.82-1.13*			
<i>p</i>	<.00001	.012	.038	<.0001
APTT STA	1.05 \pm .12	1.90 \pm .87	2.62 \pm 1.22	1.50 \pm .27
STA	.85-1.29			
<i>p</i>	<i>ns</i>	.09	.06	<.00001
APTT STA	1.06 \pm .13	1.75 \pm .61	1.94 \pm .51	2.02 \pm .38
ACL	.83-1.28			

* Normal range

TABLE 2. Heparin sensitivity index (HSI) and 95% confidence interval of the combinations including the APTT STA reagent in patients and controls or in patients only.

	HSI*	95% C.I.
Patients+		
Controls		
STA/STA	0.843	.80-.88
STA/ACL	0.999	.91-1.09
Patients		
STA/STA	0.731	.69-.78
STA/ACL	1.01	1-1.02

*Assuming a HSI of 1.0 for Actin FS/MLA

In the monitoring of oral anticoagulation, commutability of the results is of extreme importance because of the requirement for long term treatment and the possibility that patients may shift laboratories. Such a requirement would appear less stringent in the monitoring of heparin treatment, which is always carried out for a short period of time and is generally limited to in-hospital patients. However, comparability of the results is needed for the identification of optimal therapeutic ranges. Attempts to standardize the APTT test for the monitoring of heparin

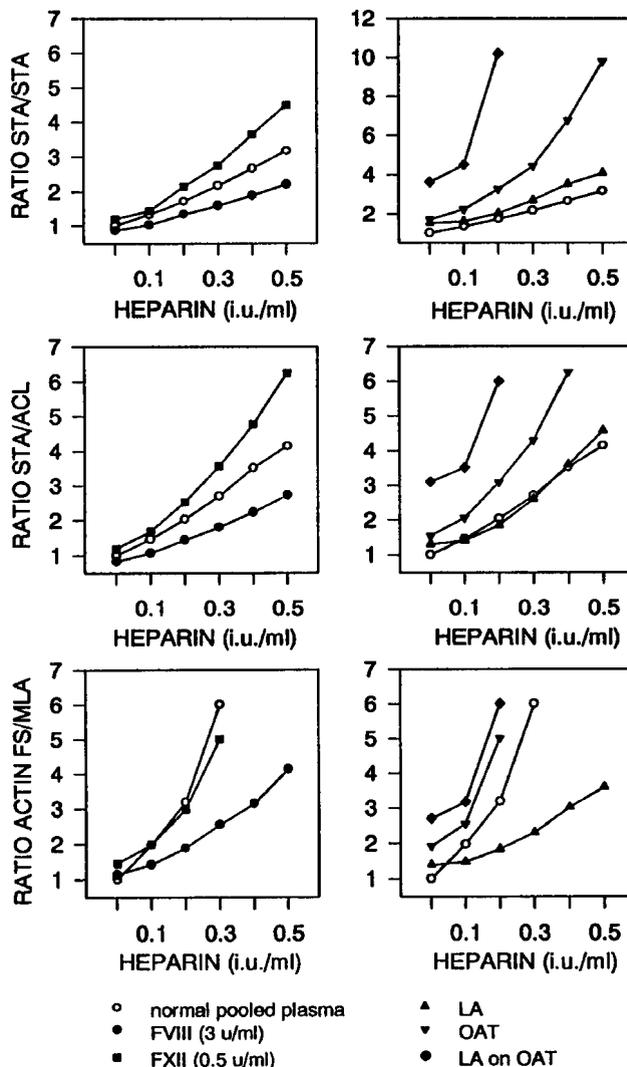


Fig. 1 In vitro heparin dose-response curves (APTT Ratio) with the three reagent-instrument combinations.

treatment are currently underway by the SSC-ISTH. Two questions are waiting for an answer; a) is standardization feasible? b) does it make sense?

It is now well established that the response of APTT reagents to the in vitro addition of heparin to normal plasma is far from reproducing the in vivo response to heparin therapy (5). Hence, attempts to standardize the APTT test in the monitoring of heparin therapy are following the lines of the INR system standardization. Obtainment of heparin sensitivity indexes (HSI) - analogous to the ISI of PT reagents (14) - can be accomplished by testing normal subjects and patients on heparin treatment with the test reagent-instrument combination and a with a reference reagent-instrument combination. We have simulated this approach arbitrarily assuming the combination of Actin FS and MLA as the reference reagent-instrument combination. The results are shown in Table 2. Markedly different HSI values were obtained by orthogonal regression analysis with the same reagent depending on the instrument used, with the additional problem of a different HSI when including both patients and normal controls or patients only in the analysis. These data suggest that in principle, the approach is feasible. However, it may not be workable in practice, because of the changes occurring during heparin treatment in the levels of clotting factors measured by the APTT. Patients requiring heparinization may differ with respect to their factor VIII and fibrinogen levels, which may

change significantly throughout treatments. Thrombosis may occur in patients with moderate deficiencies of the factors involved in the contact phase of coagulation and is frequently observed in patients with lupus anticoagulants. Last but not least, in most patients - but not in all -, oral anticoagulant treatment is overlapped to heparin treatment for 4 to 5 days, with the influence on APTT ratios resulting from the decrease in vitamin K-dependent clotting factors. These variables can hardly be taken into account by the HSI standardization model. We tried to reproduce the above clinical settings by adding heparin to: a) normal pooled plasma; b) normal pooled plasma brought to a factor VIII concentration of 3.0 u/ml by addition of a commercial factor VIII concentrate (Hemate P, Behring); c) a 1:1 mixture of normal pooled plasma with factor XII deficient plasma; d) plasma from a patient with lupus anticoagulant; e) plasma from a patient on warfarin (INR= 2.5); f) plasma from a patient with lupus anticoagulant on warfarin (INR=3.1). The in vitro heparin dose-response curves obtained with the three reagent-instrument combinations are shown in Fig. 1. The large differences observed also within the same reagent-instrument system suggest that the APTT test should be used for the screening of clinically relevant clotting factor deficiencies but not for measuring plasma heparin levels because APTT standardization is unworkable in practice. As recommended by Dr. Denson (SSC-ISTH Subcommittee Meeting, Jerusalem, June 10, 1995) heparin therapy should be monitored by measuring heparin in units by anti-Xa assays.

REFERENCES

1. A. D'Angelo. *J. Int. Fed. Clin. Chem.* **5**, 62 (1993).
2. NCCLS Document H28-T (ISBN 1-56238-167-9). *NCCLS* **12**, NO 22 (1992).
3. NCCLS Document H29-T (ISBN 1-56238-168-7). *NCCLS* **12**, NO 23 (1992).
4. A. D'Angelo *et al. Am. J. Clin. Pathol.* **92**, 321 (1989).
5. A. D'Angelo *et al. Am. J. Clin. Pathol.* **94**, 297 (1990).
6. A.M.P.H. van den Besselaar AMHP. *Thromb. Haemost.* **72**, 487 (1994).
7. A.M.H.P. van den Besselaar *et al. Am. J. Clin. Pathol.* **99**, 82 (1984).
8. L. Poller. *Biol. Clin. Haematol.* **9**, 203 (1987).
9. H. Lang *et al. Hämostaseologie* (1995), in press.
10. R.H.M. Peters *et al. Thromb. Haemost.* **66**, 442 (1991).
11. H. Lang *et al. Hämostaseologie* **13**, 96 (1993).
12. A.M.H.P. van den Besselaar. *Thromb. Haemost.* **70**, 794 (1993).
13. A. D'Angelo. *Eur. J. Clin. Chem. Biochem.* (1995), in press
14. E.A. Loeliger *et al. BCR Information* EUR 7763 EN (1984).