

Papers

Field study of lyophilised plasmas for local prothrombin time calibration in the Netherlands

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Abstract

Aim—To assess the effect of a lyophilised calibrant plasma procedure on the international normalised ratio (INR) and its interlaboratory variation.

Methods—INR equivalent values were assigned to five lyophilised plasmas (one from normal donors and four from coumarin treated patients) by a reference laboratory using three calibrated thromboplastin reagents. The calibrant plasmas and five artificial control blood specimens were mailed to 44 Dutch laboratories for prothrombin time (PT) determination. The assigned INR values were used to calculate calibration lines for each participant laboratory. The calibration lines were then used to translate the PT of the control specimens to INR.

Results—For all lyophilised plasmas and control blood samples, there were significant differences between INR values determined with the three thromboplastin reagents. These differences could not be explained by inaccuracy of the international sensitivity index or mean normal PT of the reagents and must, therefore, have been induced by the preparation procedures for the lyophilised plasmas and control blood samples. The interlaboratory variation of the INR obtained with the calibrant plasma procedure had a coefficient of variation (CV) ranging between 2.1% and 7.3% and tended to be lower than the interlaboratory variation found with the usual methods (3.0–12.2% CV). There was a good agreement between the mean INRs obtained with the calibrant procedure and those obtained using the normal methods.

Conclusions—The present study highlights the limitations of some lyophilised plasmas and control blood samples. It is not possible to assign a single INR value to each of these lyophilised plasmas and control specimens that is valid for all thromboplastin reagents. Nevertheless, by using reagent specific INR equivalent values for the calibrant plasma procedure, the interlaboratory variation could be reduced.

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The recommended scale for reporting the prothrombin time (PT) in oral anticoagulant control is the international normalised ratio (INR).^{1,2} External quality assessment schemes in various countries have demonstrated a wide range of INR interlaboratory variation.³⁻⁶ The interlaboratory variation of the PT and INR is predominantly systematic.^{4,7,8} Several investigators have tried to reduce the interlaboratory variation by providing lyophilised calibrant plasmas with assigned INR values.^{4,5,9} In some studies the test plasmas were also lyophilised and it is, therefore, not surprising that a substantial reduction of the coefficient of variation was achieved.

An external quality assessment scheme for laboratories controlling oral anticoagulant therapy has been available in the Netherlands since 1974.¹⁰ Artificially prepared blood specimens have been used because the majority of the participants used the Thrombotest (Nycomed, Oslo, Norway) reagent with citrated blood samples. Thrombotest is a combined reagent containing bovine tissue factor and adsorbed bovine plasma. A substantial number of participants are now using plain tissue factor reagents with plasma for INR determination. These participants can use the same control blood specimens, but they should be centrifuged before being tested.

In the present field study, five lyophilised plasmas were used for local PT calibration by participants of the Netherlands external quality assessment scheme. Participants were selected on the basis of the reagent used. Only laboratories using the reagents PT-Fib HS, Innovin, or Thromborel-S were included. The purpose of the study was to compare INRs calculated by the participants' usual method with INRs obtained using a lyophilised plasma calibrant procedure.

Methods**REAGENTS**

The following thromboplastin reagents were used by the author's laboratory (reference laboratory): PT-Fib HS lot I 0830341 (Instrumentation Laboratory BV, IJsselstein, The Netherlands); Innovin lot TFS-002 (Dade

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International Inc, Miami, Florida, USA); Thromborel-S lot 505557 (Behringwerke AG, Marburg, Germany).

INSTRUMENTS

The following instruments were used by the reference laboratory: ACL-300 (Instrumentation Laboratory); Electra MLA-900 (Medical Laboratory Automation, Inc, Pleasantville, New York, USA); Coagulometer according to Schnitger and Gross (H. Amelung GmbH, Lemgo, Germany).

REAGENT/INSTRUMENT CALIBRATION

The international sensitivity index (ISI) and mean normal prothrombin time (MNPT) of the reagents and instruments were determined by the reference laboratory using RBT/90 as the international reference preparation.¹¹ For a full calibration, fresh plasma specimens from 20 healthy donors and 60 patients on oral anticoagulant therapy were analysed with RBT/90 and each reagent system. Orthogonal regression lines of log transformed PT values were calculated as described previously.¹ The assigned ISI and MNPT were the mean of at least two full calibration studies.

LYOPHILISED PLASMAS

The lyophilised plasmas were prepared from normal plasma and from the plasma of patients treated with coumarin according to procedures described previously,^{12,13} with minor modifications. Blood was collected in an anticoagulant solution containing 0.109 mol/l trisodium citrate and 0.27 mol/l HEPES buffer, pH 7.3. After centrifuging twice, the plasma was stored in polypropylene tubes at -70°C . After thawing the plasmas in a waterbath at 37°C and pooling, 1 ml aliquots were measured into 14 ml siliconised vials and lyophilised at the Netherlands Public Health Institute, Bilthoven. After secondary drying over P_2O_5 , the vials were closed with rubber stops in vacuo. Each vial of lyophilised plasma was reconstituted with 1.0 ml of distilled water. The pH of the lyophilised plasmas was increased slightly due to loss of carbon dioxide during the lyophilisation process.¹⁴

Lyophilised pooled normal plasma (plasma A) was prepared from blood donations from four healthy volunteers (500 ml blood from each donor). Each of the other lyophilised calibrants was prepared by pooling 2–3 ml plasma aliquots obtained from 600–1200 patients on long term oral anticoagulant therapy. The interval between blood drawing and freezing of patient plasma was approximately 28 hours. Four different levels of coumarin effect were represented by the lyophilised plasmas B, C, D, and E, respectively. Each level was obtained by pooling individual specimens with a limited range of INR values.

At least 10 vials of each lyophilised plasma were analysed separately with the three thromboplastin reagents by the reference laboratory. Each PT was translated into INR using the formula: $\text{INR} = (\text{PT}/\text{MNPT})^{\text{ISI}}$. INR values obtained with different systems were compared with the Student's *t* test.

Table 1 International sensitivity index (ISI) and mean normal prothrombin time (MNPT) of reagents calibrated by the reference laboratory

Reagent	Instrument	ISI (SD)	MNPT (SD)
PT-Fib HS	ACL-300	1.50 (0.04)	11.9 (0.6)
Innovin	Electra-900	1.01 (0.03)	10.7 (0.2)
Thromborel-S	Electra-900	1.22 (0.07)	12.4 (0.4)
Thromborel-S	Schnitger and Gross	1.08 (0.02)	12.4 (0.4)

CONTROL BLOOD SPECIMENS

For the external quality assessment survey of February 1995, five liquid control blood specimens were prepared, one from pooled normal plasma and four from pooled plasmas of patients treated with coumarin. The pooled plasmas were prepared as described above. Different levels of coumarin effect were selected to represent the therapeutic range of anticoagulation.

Control blood specimens were prepared as described previously,¹⁵ with minor modifications. Erythrocytes were obtained from donor blood (blood group O) collected in citrate anticoagulant (CPD.A1).¹⁶ The erythrocytes were incubated with a solution containing 8-hydroxyquinoline sulphate, sodium citrate and sodium chloride (pH 6.0) for two hours at room temperature. After centrifugation, the erythrocytes were mixed with the pooled plasmas, aiming at a haematocrit of 42%, and penicillin and streptomycin were added to prevent microbial growth.

After preparation on February 6, 1995 the specimens were posted to 44 participants, together with three vials of each lyophilised plasma. Participants were instructed to analyse the lyophilised plasmas on three days, in duplicate, using their routine PT test method. On the third day (February 10) they analysed one set of lyophilised plasmas and the five control blood specimens.

CALCULATIONS

For each participant, a local calibration line was calculated by orthogonal regression of the log transformed plasma clotting times on the log transformed INR equivalents assigned by the reference laboratory using the appropriate reagent. In general, 30 data points were used for each regression analysis (five plasma calibrants were analysed on three days, in duplicate). All calculations were performed centrally to exclude any possible variation in calculation methods. Using the local calibration line, the clotting times of the blood specimens were transformed to INR equivalents.

Results

ISI and MNPT values of the reagents and instruments determined by the reference laboratory are shown in table 1. Thromborel-S was used with two different instruments. The ISI of Thromborel-S used with the MLA-900 was significantly higher than that of the same reagent used with the Schnitger and Gross instrument.

INR equivalent values for the lyophilised plasma calibrants were determined by the reference laboratory using the calibration

Table 2 Mean INR equivalent values (SD) for lyophilised calibrant plasmas determined by the reference laboratory using different reagent systems

Calibrant plasma	pH	PT-Fib HS ACL-300	Innovin Electra	Thromborel-S Electra	Thromborel-S Schnitger
A	7.9	1.12 (0.03)	1.12 (0.03)	1.04 (0.02)	1.03 (0.02)
B	7.5	2.32 (0.05)	1.80 (0.07)	2.04 (0.10)	2.02 (0.06)
C	7.9	3.02 (0.06)	2.18 (0.11)	2.59 (0.24)	2.62 (0.06)
D	8.0	4.75 (0.18)	3.45 (0.19)	4.05 (0.43)	4.18 (0.12)
E	8.0	5.78 (0.24)	4.48 (0.24)	5.02 (0.43)	5.26 (0.14)

pH values were measured by the reference laboratory.

Table 3 Number of participants using various reagent/instrument combinations

Instrument	PT-Fib HS	Innovin	Thromborel-S
ACL (IL)	21	1	0
Electra (MLA)	0	5	4
KC (Amelung)	0	6	0
Schnitger and Gross	0	1	0
Amax (Amelung)	0	1	1
Hemolab (BioMérieux)	0	1	0
BFA (Behring)	0	0	1

parameters given in table 1. For plasma A, there was no difference between INR with PT-Fib HS and Innovin (table 2). For all other lyophilised plasmas, the INRs with PT-Fib HS were significantly higher than those with Thromborel-S, and the INRs with Thromborel-S were all higher than those with Innovin (table 2). There was no difference between the INRs determined with Thromborel-S on the two instruments.

Forty two laboratories in the Netherlands returned results. The instruments used by the participants are given in table 3. Orthogonal regression analysis was used to determine the relationship between log INR (determined by the reference laboratory) and log PT (determined by a field laboratory) on the five calibrant plasmas. In all cases the correlation coefficients were greater than 0.993. Only one field laboratory noted an obviously outlying measurement for calibrant A on the second day, which was excluded from the regression analysis. The orthogonal regression lines were used to translate the PTs of the control

Table 4 Mean INR equivalent values and interlaboratory coefficient of variation (CV) for control blood samples calculated with the calibrant plasma procedure

Sample	PT-Fib HS (n=21)	Innovin (n=15)	Thromborel-S (n=6)
	mean (CV%)	mean (CV%)	mean (CV%)
1	1.39 (5.0)	1.04 (3.4)	1.35 (2.1)
2	3.39 (6.9)	2.17 (4.2)	3.08 (4.7)
3	4.76 (7.3)	2.88 (3.6)	4.25 (5.4)
4	2.63 (5.8)	1.68 (3.0)	2.42 (4.8)
5	3.43 (6.6)	2.15 (4.0)	3.08 (6.8)

Table 5 Mean INR equivalent values and interlaboratory coefficient of variation (CV) for control blood samples calculated by participants using their usual method

Sample	pH	PT-Fib HS (n=20)	Innovin (n=6)	Thromborel-S (n=6)
		mean (CV%)	mean (CV%)	mean (CV%)
1	7.1	1.40 (5.5)	1.13 (6.8)	1.33 (9.1)
2	7.0	3.31 (7.8)	2.29 (6.0)	3.08 (8.9)
3	7.0	4.60 (8.4)	2.94 (4.6)	4.35 (12.2)
4	7.0	2.59 (6.8)	1.80 (3.0)	2.40 (9.0)
5	7.0	3.36 (7.6)	2.25 (4.1)	3.11 (11.3)

pH values were measured by the reference laboratory.

specimens into INRs. The mean INRs and the interlaboratory variation are shown in table 4. Obviously, the INR equivalents calculated with PT-Fib HS and Thromborel-S are greater than those with Innovin. For comparison, the mean INRs and associated interlaboratory variation of the same control specimens determined by participants' routine methods (not specified) are given in table 5. Several participants using Innovin failed to determine and report INRs with their usual procedure (tables 4 and 5). The differences in mean INR between the calibrant method and the participants' routine methods were relatively small, ranging from 0–8.7%. There was a tendency towards a lower interlaboratory variation with the calibrant method compared with the routine methods.

Discussion

The INR is defined as the normalised PT ratio calculated for a fresh plasma specimen from a patient stabilised on long term oral anticoagulant treatment. The mean INR of a sufficiently large group of stabilised patients should be independent of the PT reagent and instrument used for the assessment. However, the INR equivalent values of the lyophilised plasma calibrants used in this study were dependent on the thromboplastin reagent used: the values determined with PT-Fib HS were higher than those with Thromborel-S and Innovin (table 2). These differences cannot be explained by uncertain ISI values because ISI and MNPT were determined by the same reference laboratory with good precision (table 1). Therefore, these lyophilised plasmas must react differently to fresh plasma specimens, although the mechanism responsible for the bias is not known. The production of lyophilised pooled plasma from fresh specimens took a long time and involved one freeze/thaw cycle before the eventual lyophilisation procedure. During this long process, partial activation or inactivation of individual coagulation factors may have occurred. Significant differences between INRs of lyophilised plasmas determined with different reagents have been noted in other studies.^{6, 17–19} If lyophilisation is a cause of INR discrepancies between reagents, the use of deep-frozen plasma calibrants may improve the accuracy of local calibration. This should be investigated in future studies.

Whatever the cause of the differences may be, it is not possible to assign a single INR value to each of these lyophilised plasmas for local ISI calibration. There is no "true" value, valid for all reagents. Using a single value (for example, the mean of all determinations with all reagents) would lead to greatly inaccurate INRs for several reagents with adverse effects on the clinical dosage of anticoagulants. For this reason the local calibration lines were based on reagent specific INR values—for example, log PT determined by a participant using PT-Fib HS was related to log INR determined by the reference laboratory using the same reagent. The local PT determinations were performed on three subsequent days because calibration on one day only appeared

to be less precise.^{5,20} A three day local calibration resulted in excellent correlation coefficients (> 0.993) in all laboratories.

There were large and consistent differences between the INRs for the control blood samples determined with the three reagents (tables 4 and 5). Similar differences had been noted in previous external quality assessment surveys. The differences cannot be explained by ISI or MNPT errors and, therefore, must be due to the method of preparation. Control blood samples had a pH of ~ 7.0 (table 5) due to the relatively low pH of red blood cells after treatment with the 8-hydroxyquinoline sulphate solution. Lowering of the pH induces a prolongation of the PT.^{14,21} Recent investigations showed that the magnitude of this prolongation is different for the various reagents (van den Besselaar, unpublished data), explaining part of the INR differences reported in tables 4 and 5. The preparation of control blood for the Netherlands external quality assessment scheme may be improved to reduce the INR differences between reagents.

The mean INRs of the control specimens determined with the calibrant procedure (table 4) were in good agreement with the corresponding values determined by the participants' usual procedures (table 5). This indicates that, on average, there is a good agreement between the calibration by the reference laboratory (on which the assigned calibrant values are based) and the manufacturers' calibration (on which the participants' usual values are based).

Despite the fact that the average values were in good agreement, there was considerable interlaboratory variation with the usual procedures (table 5). This variation could be reduced substantially with the lyophilised calibrant procedure, particularly for Thromborel-S. A moderate reduction of the interlaboratory variation was observed for PT-Fib HS. The level of interlaboratory variation remaining after local calibration in the present study is similar to the level observed with Thrombotest in a previous study.⁴

In conclusion, the present study highlights the limitations of lyophilised plasma calibrants and external quality assessment control blood samples. The necessary improvements might be achieved by using deep-frozen calibrants and adjusting the pH of the control blood samples.

Despite the limitations, local calibration by means of lyophilised plasma calibrants may reduce interlaboratory variation of the INR to an acceptable level, provided that the values assigned to the calibrants are carefully checked by a reference laboratory using the same reagents as the field laboratories.

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