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Stability of coagulation assays performed in plasma from citrated whole blood transported at ambient temperature

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Summary

Many preanalytical variables affect the results of coagulation assays. A possible way to control some of them would be to accept blood specimens shipped in the original collection tube. The aim of our study was to investigate the stability of coagulation assays in citrated whole blood transported at ambient temperature for up to two days after specimen collection. Blood samples from 59 patients who attended our haematology outpatient ward for thrombophilia screening were transported at ambient temperature (outdoor during the day, indoor overnight) for following periods of time: <1 hour, 4–6, 8–12, 24–28 and 48–52 hours prior to centrifugation and plasma-freezing. The following coagulation tests were performed: PT, aPTT, fibrinogen, FII:C, FV:C, FVII:C, FVIII:C, FIX:C, FX:C, FXI:C, VWF:RCo, VWF:Ag, AT, PC activity, total and free PS antigen, modified APC-sensitivity-ratio, thrombin-antithrombin-complex and D-dimer. Clinically significant changes, defined as a percentage change of more than 10% from the initial value, were observed for FV:C, FVIII:C and total PS antigen starting at 24–28 hours, and for PT, aPTT and FVII:C at 48–52 hours. No statistically significant differences were seen for fibrinogen, antithrombin, or thrombin-antithrombin complexes (Friedman repeated measures analysis of variance). The present data suggest that the use of whole blood samples transported at ambient temperature may be an acceptable means of delivering specimens for coagulation analysis. With the exception of factor V and VIII coagulant activity, and total PS antigen all investigated parameters can be measured 24–28 hours after specimen collection without observing clinically relevant changes.

Keywords

Coagulation factors, thrombophilia, laboratory, preanalytic

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Introduction

Preanalytical conditions are of prime importance in laboratory assessment of haemostasis. Many variables such as blood collecting system, anticoagulant type and concentration, haematocrit and filling status of the sampling tube, transport, centrifugation and storage of the blood specimen affect the results of coagulation assays. For central coagulation laboratories serving external hospitals and practising physicians, a possible way to control for some of those variables would be to accept blood specimens transported at ambient temperature as citrated whole blood in the original collection tube. The fourth edition of the CLSI (formerly NCCLS) guideline for "Collection, Transport, and Processing of Blood Specimens for Testing Plasma-Based Coagulation Assays" (1) states that: "A. Specimens for prothrombin time (PT) assays uncentrifuged […] in an unopened tube kept at 18–24°C should be tested within 24 hours from time of specimen collection; B. Specimens for routine aPTT (activated partial thromboplastin time) assays on non-heparinized patients uncentrifuged […] in an unopened tube kept at 2–4°C or 18–24°C should be tested within 4 hours from time of specimen collection; and C. Specimens for other assays […] kept at 2–4°C or 18–24°C should be centrifuged and tested within 4 hours from time of specimen collection" (1). Even though it has been loosened compared to the third edition, this guideline – stating a time limit of 4 hours (h) for non-heparin aPTT and "other assays" – still poses significant constraints to centralized coagulation laboratories. While PT measurements appear to be stable in plasma from whole blood stored up to 24 h as indicated by CLSI, non-heparin aPTT results have been described to be stable for longer periods of time than requested by the guideline, at least up to 8 h (2, 3). In addition, to our knowledge, the stability of single coagulation factors in whole blood stored for variable time periods has not been evaluated systematically, and the stability of

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natural anticoagulants (4) and activation markers of coagulation has been examined by few studies only (4, 5).

The aim of our work was to investigate the stability of coagulation assays in citrated whole blood transported at ambient temperature for up to two days after specimen collection.

Patients and methods

Blood sample collection and processing

We investigated plasma samples of 59 patients who attended our haematology outpatient ward for thrombophilia screening (49 in November-December and 10 in July-August). Twelve patients were anticoagulated with vitamin K antagonists (VKA). None of them received heparin. After informed consent was given, blood was collected by standard venepuncture into 0.106 M tri-sodium citrate (9:1 vol/vol) in plastic syringes (Monovette, Sarstedt, Nümbrecht, Germany). From each patient we collected five tubes for coagulation analysis. The first tube was routinely sent to the laboratory by pneumatic mail and centrifuged within 1 h. The additional whole blood samples were transported for following lengths of time: 4–6 h, 8–12 h , 24–28 h and 48–52 h at ambient temperature and were then centrifuged in random order of draw. Platelet-poor plasma (PPP) containing less than 10,000 platelets per µl was prepared by double centrifugation at 1,500 g for 10 minutes (min) each at 20°C. PPP samples were aliquoted, snap-frozen and stored at –80°C until testing. Freezing of the PPP samples at time <1 h was performed by routine laboratory technicians, at all other time points by the first author. Before testing, the plasma samples were thawed in a water bath at 37°C for 5 min and then briefly vortexed. The investigational protocol had been approved by the competent review board (Kantonale Ethikkomission Bern, University of Berne, Switzerland).

Blood sample transportation

Blood samples were transported during the day by the first author in his backpack and were stored indoor overnight. The median daytime (7 a.m. – 7 p.m.) outdoor temperature during the winter period was 2°C (min. –12°C, max. 10°C) and during the summer period 17°C (min. 11°C, max. 29°C). Overnight indoor temperature was 20–25°C. Outdoor temperature was hourly measured in Bern-Liebefeld by MeteoSchweiz (Zurich, Switzerland) and data were kindly provided by MeteoTest (Berne, Switzerland).

Effect of plasma freezing

In order to detect variations due to transportation time, the five specimens of a single patient had to be measured batch-wise in frozen samples. In a pilot phase we therefore investigated the effect of the freezing/thawing procedure on those parameters, which we routinely assess in fresh PPP. We found a statistically significant difference for aPTT (median value in fresh PPP 30.6 seconds [sec] vs. thawed PPP 33.0 sec; n=10, p=0.002, Wilcoxon signed rank-test) and for FV:C (128% vs. 100%; n=10, p=0.002). For aPTT, the median prolongation by the freezing/thawing procedure was 8% (i.e. $aPTT_{thawed} = 1.08 * aPTT_{fresh}$) and the relation derived by linear regression was: $aPTT_{thawed} = -16.930 + 1.702 * aPTT_{fresh}$ (n=10; r^2=0.969; standard error of estimate 4.502). For FV:C, the median activity decline was 22% (i.e. $FV{:}C_{thawed} = 0.78 * FV{:}C_{fresh}$) and the equation by linear regression was: $FV{:}C_{thawed} = 5.055 + 0.742 * FV{:}C_{fresh}$ (n=10; r^2=0.901; standard error of estimate 5.212). All other analyses, PT (p=0.125), fibrinogen (p=0.232), FII:C (p=0.064), FVII:C (p=0.570), FX:C (p=0.844) and D-dimer (p=0.734) were not affected by the freezing/thawing procedure (n=10).

When we analyzed the results from the winter part of the study we found a peculiar behaviour for aPTT, FV:C and FVIII:C. At first sight, Supplemental Data Figure 1A (see online at www.thrombosis-online.com) for aPTT and FV:C appeared to show the expected changes between results in fresh PPP compared to thawed samples at time <1 h. However, the aPTT decrease and the increase of FV:C and FVIII:C observed in specimens transported for longer periods of time were puzzling. The solution to this riddle came when we realised that PPP from the first specimen (time <1 h) happened to be left capped on the bench for up to 2–3 h before aliquotation and freezing. Laboratory standards were improved and freezing of PPP from time <1 h in the summer part of the study was performed immediately after centrifugation (Supplemental Data Fig. 1B; see online at www.thrombosis-online.com). Results at later time points were not affected, since centrifugation, aliquotation and immediate snap-freezing were always performed by the first author. Parameters other than aPTT, FV:C and FVIII:C were not relevantly altered and original results from the frozen samples at time <1 h are presented in the tables. Values for aPTT (Table 2) and FV:C (Table 1) at time <1 h are calculated from the corresponding routine results corrected by the freezing/thawing effect (see above). For aPTT we employed the equation $aPTT_{thawed} = 1.08 * aPTT_{fresh}$ because it gave a more conservative estimate for aPTT at time < 1 h than the relation derived by linear regression. For FV:C, both formulas gave the same estimates.

Reagents and instruments

All laboratory methods, with the exception of VWF:Ag, employed commercial kits. A detailed description of the employed laboratory methods is offered online (see Supplemental Data "Laboratory methods" online at www.thrombosis-online.com). See *Appendix* for between-run coefficients of variation (CV) in our laboratory.

Statistical analysis

Analysis of variance between the various time intervals for a given assay was performed by both Kruskal-Wallis-test and Friedman repeated measure analysis of variance. Statistically significant differences compared to the control group (time <1 h) were assessed by the Dunn's method. In addition, the percentage change between the control value and subsequent time points was calculated using following equation:

$$\%Change_{t=x} = (Result_{t=x} - Result_{t=<1hr})/Result_{t=<1hr} \times 100$$

The 99%-confidence interval (99%-CI) for the percentage change at every time point was calculated as follows:

$$Mean\ \%Change \pm (t_{0.01} \times SEM),$$

were $t_{0.01}$ is the percentage point of the *t*-distribution with (n-1) degrees of freedom which gives a two-tailed probability of 0.01 and SEM is the standard error of the mean. Finally, a clinically relevant difference was conservatively defined as a change in the coagulation test value of greater than 10% compared to the

Table 1: Prothrombin time and single coagulation factors.

Time (h)	PT (n = 59)				FII:C (n = 59)				FV:C (n = 59)			
	Activity (% NHP)		Change vs. <1h (%)		Activity (% NHP)		Change vs. <1h (%)		Activity (% NHP)		Change vs. <1h (%)	
	Median	(IQR)	Mean	(99%-CI)	Median	(IQR)	Mean	(99%-CI)	Median	(IQR)	Mean	(99%-CI)
<1	100	87 – 109			105	93 – 126			105 [1)]	91 – 116		
4–6	101	88 – 110	2.5	0.7 – 4.3	108 †	90 – 130	2.3	0.2 – 4.4	105	93 – 125	2.8	–0.6 – 6.2
8–12	99	88 – 110	2.5	0.4 – 4.6	110 †	93 – 126	3.1	0.8 – 5.3	104	94 – 122	2.6	–1.1 – 6.3
24–28	94 †	74 – 103	– 4.2	–6.9 – –1.6	110 †	93 – 129	2.7	0.0 – 5.5	91 *†	76 – 104	– 12.4 ‡	–17 – –7.9
48–52	84 *†	61 – 96	– 10.8 ‡	–14.8 – –6.8	108 †	90 – 125	2.6	0.1 – 5.0	75 *†	60 – 97	– 27.0 ‡	–33 – –22
P-value												
Kruskal-Wallis	<0.001				0.951				<0.001			
Friedman	<0.001				<0.001				<0.001			

NHP: Normal human plasma. IQR: Inter-quartile range. [1)]: Calculated values according to the equation FV:C $_{thawed}$ = 0.78 * FV:C $_{fresh}$ (see Methods section for details). * denotes a statistically significant difference (p<0.05) compared to the control group (time <1hour) following Kruskal-Wallis One Way ANOVA on ranks. † denotes a statistically significant difference (p<0.05) compared to the control group (time <1hour) following Friedman Repeated Measures ANOVA on ranks. ‡ denotes a potentially clinically significant difference (> 10%) of the 99%-CI for the percentage change compared to the control group (time < 1hour).

Time (h)	FVII:C (n = 59)				FX:C (n = 59)				Fibrinogen (n = 59)			
	Activity (% NHP)		Change vs. <1h (%)		Activity (% NHP)		Change vs. <1h (%)		Clauss (g/l)		Change vs. <1h (%)	
	Median	(IQR)	Mean	(99%-CI)	Median	(IQR)	Mean	(99%-CI)	Median	(IQR)	Mean	(99%-CI)
<1	112	77 – 136			111	86 – 127			3.06	2.6 – 3.5		
4–6	108	74 – 133	– 1.2	–2.4 – 0.0	113 †	85 – 131	3.0	0.9 – 5.2	3.06	2.7 – 3.5	0.4	– 1.0 – 1.9
8–12	111	78 – 136	– 1.0	–2.5 – 0.5	112 †	87 – 134	4.0	1.5 – 6.6	3.07	2.7 – 3.5	0.8	– 1.4 – 3.0
24–28	103 †	71 – 130	– 8.2	–9.8 – –6.7	110	85 – 130	1.7	–0.6 – 3.9	3.11	2.7 – 3.5	0.8	– 0.9 – 2.5
48–52	97 †	67 – 126	– 12.8 ‡	–15 – –11	110	83 – 128	0.3	–1.9 – 2.4	3.17	2.7 – 3.5	0.1	– 2.4 – 2.7
P-value												
Kruskal-Wallis	0.230				0.960				0.999			
Friedman	<0.001				<0.001				0.085			

control value. All statistical analyses were performed with SigmaStat (version 3.1, Jandel Scientific, San Rafael, CA, USA).

Results

Global coagulation assays and single factors

Since a comparison of the winter versus the summer data did not reveal statistically significant differences for percent changes over time for any single parameter measured, results were pooled. Table 1 shows that when PPP is prepared ≥24 h after venepuncture a statistically significant decline of the values for prothrombin time (PT) given in Quick percentage is observed. However, the percentage change from the initial measurement is less than 10% (99%-Cl: –6.9 – –1.6%) at 24 h and less than 15% (99%-CI: –14.8 – –6.8%) at 48 h. The change in PT values is primarily due to a significant decline of factor V:C at 24 h (mean decline: 12.4%) and 48 h (mean decline: 27.0%), and additionally to a decline in factor VII:C at 48 h (mean decline: 12.8%). Factor II:C, factor X:C and fibrinogen remain stable up to 48 h after blood drawing. Figure 1A depicts the course of PT measurements for patients with initial PT values within the low-normal range (70–100%) and patients anticoagulated with vitamin K antagonists. It appears that the PT percent-activity starts declining at 24 h in blood samples with low-normal values, while it remains stable in anticoagulated blood samples. Figure 1B shows the time course of INR for anticoagulated patients: median INR values were 2.74 at time <1 h, 2.43 at 4–6 h, 2.47 at 8–12 h, 2.58 at 24–28 h, and 2.65 at 48–52 h (p-value Kruskal-Wallis: 0.952; p-value Friedman: <0.001, with statistically significant differences between INR at 4–6 h and 8–12 h compared to the initial value). The 99%-CI for the percentage changes between the initial INR and values at 4–6 hours was –10.8 – 6.9%, and values at 48–52 hours was –5.4 – 5.5%.

Table 2 shows a similar trend for the aPTT as for PT. A statistically significant change of aPTT measurements can be observed when PPP is prepared ≥24 h after venepuncture. The percentage prolongation of the aPTT compared to the initial value is less than 10% (99%-Cl: 2.9 – 8.3%) at 24 h and less than 15% (99%-CI: 7.2 – 13.2%) at 48 h. The change in aPTT measurements is primarily due to a significant decline of factors VIII:C (Table 2) and V:C (Table 1). The other haemostatically relevant coagulation factors – factors IX:C and XI:C – are stable for at

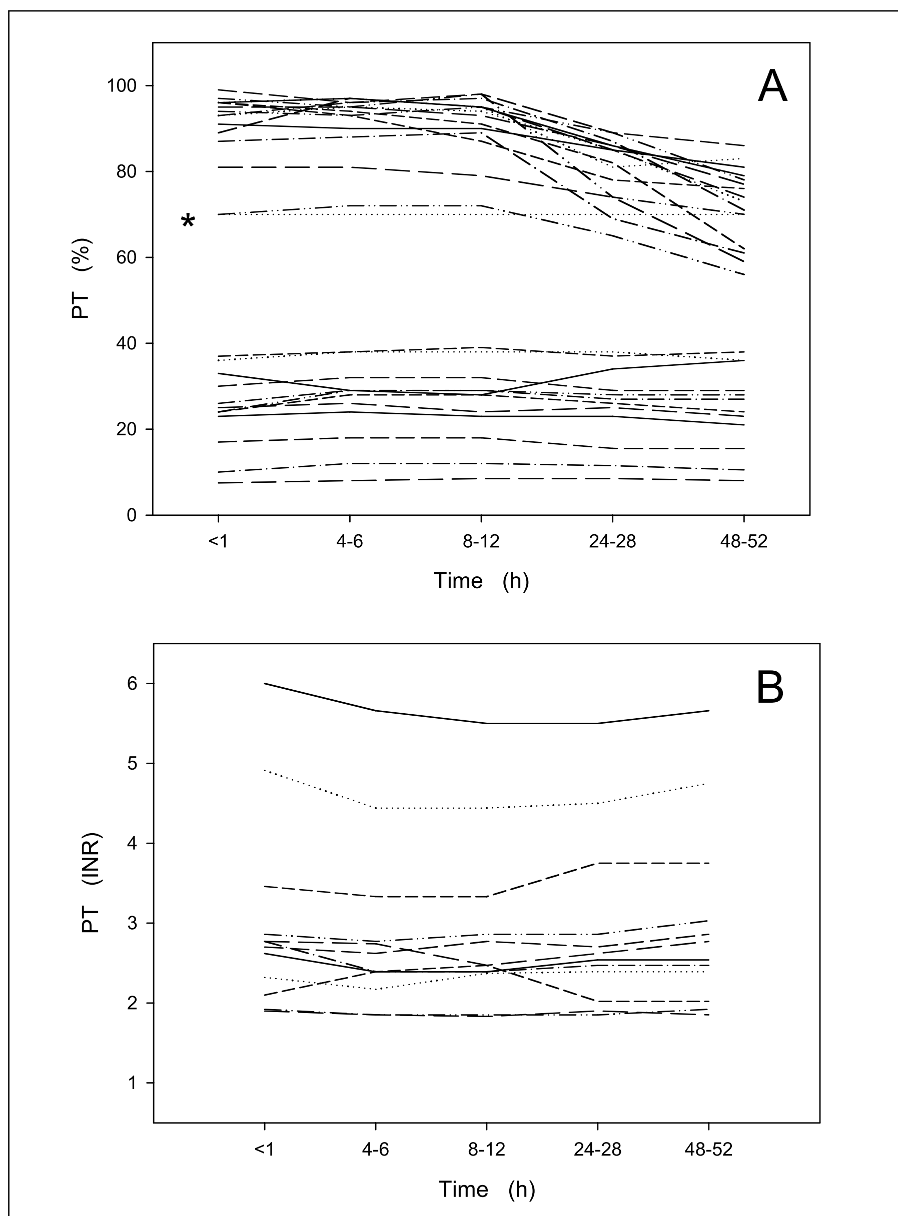

Figure 1: Prothrombin time. A) Quick percent value. Time course of all 15 samples with initially low-normal PT values (70–100%) and of all 12 samples from patients anticoagulated with vitamin K antagonists. The straight dotted line (*) represents the lower limit of the normal range (70%). B) INR value. Time course of INR values of all 12 samples from patients anticoagulated with vitamin K antagonists.

least 48 h. Concerning the von Willebrand factor (VWF), both antigen and ristocetin-cofactor activity remain stable up to 48 h, as well (Table 2).

In real-life routine analyses, such as PT, aPTT, FII:C, FV:C, FVII:C, FX:C, fibrinogen, D-Dimers are performed without plasma freezing. Table 3 shows that when citrated whole blood is transported at ambient temperature for 24 or 48 h and PPP subsequently analyzed without freezing/thawing, the changes are similar to those observed in the context of the main study (where samples were snap-frozen in order to perform batch analysis).

Natural anticoagulants

Table 4 shows that all three natural anticoagulants – antithrombin (AT), protein C (PC) and free protein S (PS) – remain stable in whole blood up to two days after venepuncture, with a 99%-CI for the percentage change from the initial value of less than 10% at 48 hours in each case. In order to visualize the risk of misclassification of individual patients, Figures 2A-F depict the course of single borderline probes. It is interesting to note that in some cases AT activity appears to increase during transportation of whole blood at ambient temperature, with the potential of missing patients with slightly decreased values (Fig. 2A). Although the overall changes in PC by chromogenic and coagulometric methods are similar (Table 4), measurement by chromogenic activity appears to be fairly precise (Fig. 2B) while the coagulometric assay is more variable (Fig. 2C; see also *Appendix* for between-run CV). A striking random variation is similarly observed for free PS antigen, particularly in women (Fig. 2D-E).

The assessment of resistance to activated PC (APC) by the modified APC sensitivity ratio shows a statistically significant decline by 48 h with both analysis of variance tests (Table 4). However, Figure 2F demonstrates that samples with heterozy-

Table 2: aPTT and single coagulation factors.

Time (h)	aPTT (n = 59)				FXI:C (n = 59)				FIX:C (n = 59)			
	Time (sec)		Change vs. <1h (%)		Activity (% NHP)		Change vs. <1h (%)		Activity (% NHP)		Change vs. <1h (%)	
	Median	(IQR)	Mean	(99%-CI)	Median	(IQR)	Mean	(99%-CI)	Mean	(IQR)	Mean	(99%-CI)
<1	32.2 [1)]	29.5 – 35.0			108	97 – 116			98	77 – 119		
4–6	32.8 †	31.0 – 36.9	3.9	1.2 – 6.5	110	99 – 115	0.7	–1.1 – 2.5	105 †	79 – 123	3.8	1.9 – 5.7
8–12	32.5	30.4 – 36.7	3.0	0.4 – 5.7	109	98 – 115	1.0	–0.9 – 2.8	104 †	81 – 123	4.1	1.8 – 6.4
24–28	34.7 †	31.6 – 38.0	5.6	2.9 – 8.3	109	100 – 115	0.2	–1.6 – 2.1	102 †	81 – 119	3.3	0.3 – 6.4
48–52	35.3 *†	32.8 – 38.9	10.2 ‡	7.2 – 13.2	109 †	99 – 117	0.9	–0.8 – 2.6	100	79 – 120	1.5	–1.5 – 4.5
P-value												
Kruskal-Wallis	0.004				0.995				0.975			
Friedman	<0.001				0.023				<0.001			

NHP: Normal human plasma. IQR: Inter-quartile range. [1)]: Calculated values according to the equation $aPTT_{thawed} = 1.08 * aPTT_{fresh}$ (see Methods section for details). * denotes a statistically significant difference (p<0.05) compared to the control group (time <1hour) following Kruskal-Wallis One Way ANOVA on ranks. † denotes a statistically significant difference (p<0.05) compared to the control group (time <1hour) following Friedman Repeated Measures ANOVA on ranks. ‡ denotes a potentially clinically significant difference (> 10%) of the 99%-CI for the percentage change compared to the control group (time < 1hour).

Time (h)	FVIII:C (n = 10)				VWF:Ag (n = 59)				VWF:RCo (n = 59)			
	Activity (% NHP)		Change vs. <1h (%)		Antigen (% NHP)		Change vs. <1h (%)		Activity (% NHP)		Change vs. <1h (%)	
	Median	(IQR)	Mean	(99%-CI)	Median	(IQR)	Mean	(99%-CI)	Median	(IQR)	Mean	(99%-CI)
<1	165	130 – 172			111	88 – 145			132	100 – 160		
4–6	146	119 – 152	– 7.3 ‡	–25 – 11	114	87 – 151	2.1	–3.9 – 8.1	132 †	101 – 165	2.5	0.4 – 4.6
8–12	135	113 – 146	– 11.6 ‡	–25 – 1.3	117 †	87 – 146	0.2	–6.1 – 6.5	134 †	104 – 168	3.8	1.5 – 6.0
24–28	110 *†	97 – 122	– 26.9 ‡	–36 – –18	116	89 – 145	0.8	–2.8 – 4.4	135 †	108 – 170	4.7	2.1 – 7.3
48–52	102 *†	87 – 109	– 32.5 ‡	–42 – –23	114	90 – 151	1.1	–2.5 – 4.6	137 †	104 – 168	5.5	2.4 – 8.7
P-value												
Kruskal-Wallis	<0.001				0.997				0.951			
Friedmann	<0.001				<0.001				<0.001			

gous factor V Leiden mutation maintain stable ratios for the whole study period (p-value Kruskal-Wallis: 0.437; p-value Friedman: 0.002). Moreover, samples with a modified APC sensitivity ratio in the low-normal range (2.2–2.5) did not drop in a clearly pathologic range.

Markers of in-vivo thrombin generation

Table 5 summarizes results for the thrombin-antithrombin-complex (TAT) and D-dimer. There is no statistically significant difference for TAT values during the study period. D-dimer show significantly higher values at 48–52 h, with a mean increase of 9.4% (99%-CI: 3.5 – 15.0%) compared to the initial results. Since D-dimer assays are mostly employed for excluding venous thromboembolism with a cut-off level of 500 µg/l, we analysed the results in terms of concordant tests: 47 patients had negative/negative results (time <1 h / 48–52 h), 10 patients positive/positive results, one patient a negative/positive discordant result (498 vs. 504 µg/l) and one patient a positive/negative mismatch (570 vs. 434 µg/l). Within our study population this would give a rate of false positive results of ≈2% and of false negative of ≈9% when D-dimer testing is performed two days instead of immediately after blood sampling.

Haemolysis

In order to assess the degree of in-vitro haemolysis, LDH and free plasma haemoglobin were measured in the initial samples and in those transported for 48–52 h. Although both LDH (264 ± 50 U/l vs. 398 ± 72; p≤0.001) and free plasma haemoglobin (0.251 ± 0.137 g/l vs. 0.371 ± 0.134 g/l; p=0.005) significantly increase, they still are within normal ranges (LDH < 480 U/l; free plasma haemoglobin < 0.5 g/l) after two days.

Discussion

The aim of the present study was to investigate the stability of citrated whole blood samples transported without cooling prior to preparation of PPP for coagulation analysis. This information is necessary to evaluate whether it is possible to ship uncentrifuged blood samples to a central laboratory. If feasible, this simple practice would allow to reduce the number of preanalytical variables which are not controlled by the laboratory itself (e.g. anticoagulant, tube filling, haematocrit, centrifugation).

Since there are no guidelines defining acceptable percentage changes compared with reference values for preanalytical variables of coagulation assays, we decided to consider a percentage

Table 3: Routine analyses performed in fresh samples.

Time (h)	PT (n = 10)				aPTT (n = 10)				FII:C (n = 10)			
	Activity (% NHP)		Change vs. <1h (%)		Activity (sec)		Change vs. <1h (%)		Activity (% NHP)		Change vs. <1h (%)	
	Median	(IQR)	Mean	(99%-CI)	Median	(IQR)	Mean	(99%-CI)	Median	(IQR)	Mean	(99%-CI)
<1	101	93 – 103			30.8	28.1 – 33.9			98	96 – 115		
24	105 †	98 – 108	4.5	3.1 – 5.9	32.3 †	29.4 – 35.0	4.2	2.8 – 5.6	102	100 – 122	3.0	–2.6 – 8.5
48	100	92 – 103	0.9	–2.5 –4.4	33.4 †	30.0 – 36.2	7.7 ‡	5.0 – 10.5	95	93 – 108	–3.9	–8.9 – 1.2
P-value												
Kruskal-Wallis	0.518				0.305				0.289			
Friedman	<0.015				<0.001				0.015			

* denotes a statistically significant difference (p<0.05) compared to the control group (time <1hour) following Kruskal-Wallis One Way ANOVA on ranks. † denotes a statistically significant difference (p<0.05) compared to the control group (time <1hour) following Friedman Repeated Measures ANOVA on ranks. ‡ denotes a potentially clinically significant difference (> 10%) of the 99%-CI of the percent change compared to the control group (time < 1hour).

Time (h)	FV:C (n = 10)				FVII:C (n = 10)				FX:C (n = 10)			
	Activity (% NHP)		Change vs. <1h (%)		Activity (% NHP)		Change vs. <1h (%)		Activity (% NHP)		Change vs. <1h (%)	
	Median	(IQR)	Mean	(99%-CI)	Median	(IQR)	Mean	(99%-CI)	Median	(IQR)	Mean	(99%-CI)
<1	121	103 – 137			117	100 –127			117	111 – 129		
24	129	111 – 143	6.8 ‡	2.0 – 11.5	113	96 – 120	–0.5	–6.1 – 5.1	114	104 – 126	–1.5	–5.4 – 2.5
48	106 †	96 – 126	–6.1 ‡	–12.3 – 0.0	105 †	89 – 114	–7.2 ‡	–12.6 – –1.7	110 †	98 – 118	–5.9	–9.6 – –3.0
P-value												
Kruskal-Wallis	0.300				0.530				0.533			
Friedman	<0.001				0.02				0.006			

Time (h)	Fibrinogen (n = 10)				D-dimer (n = 10)			
	Clauss (g/l)		Change vs. <1h (%)		Quantity (µg/l)		Change vs. <1h (%)	
	Median	(IQR)	Mean	(99%-CI)	Median	(IQR)	Mean	(99%-CI)
<1	2.79	2.3 – 3.3			193	125 – 322		
24	2.89	2.5 – 3.6	4.8	0.6 – 9.1	198	131 – 346	4.7	–6.4 – 15.4
48	3.00 †	2.5 – 3.8	9.0 ‡	4.0 – 14.0	185	137 – 355	2.4	–6.1 – 10.8
P-value								
Kruskal-Wallis	0.349				0.939			
Friedman	<0.001				0.670			

change >10% from the initial measurement to be potentially clinically relevant for following reasons. First, for calibration procedures and for testing of local PT systems, a deviation of the INR of >10% of the reference value is defined as clinically important (6, 7). Second, a 10% change represents roughly two times the average coefficient of variation (CV) of coagulation assays in our laboratory (see *Appendix*).

We show that both global coagulation assays – PT and non-heparin aPTT – can be performed 24–28 h after blood sampling without clinically relevant alterations (Tables 1, 2 and 3). These data replicate previously published results. For instance, Adcock et al. demonstrated that PT measurements in whole blood stored at room temperature showed a prolongation of the clotting time of 2.2% on average (min. –10.7%, max. 11.9%) after 24 h, and that aPTT values changed by 4.1% (min. –2.5%, max. 12.7%) at 8 h (2). Rao et al. concluded that whole blood samples transported at room temperature can be accepted up to 24 h, and probably even 48 h, for PT testing, and up to 18 h for aPTT measurement (3). In addition, our results are also in line with previous publications showing that – at least with the employed blood sampling/testing system (8) – for patients receiving vitamin K antagonists, International Normalised Ratio (INR) values are stable up to 24 h in whole blood samples (9). While all these observations are consistent with the current CLSI guideline for PT testing (1), it appears that the stated time limit of 4 h for performing non-heparin aPTT measurements is too stringent.

Table 4: Natural anticoagulants.

Time (h)	Antithrombin (n = 59)				Protein C (chromogenic) (n = 59)				Protein C (coagulometric) (n = 59)			
	Activity (% NHP)		Change vs. <1h (%)		Activity (% NHP)		Change vs. <1h (%)		Activity (% NHP)		Change vs. <1h (%)	
	Median	(IQR)	Mean	(99%-CI)	Median	(IQR)	Mean	(99%-CI)	Median	(IQR)	Mean	(99%-CI)
<1	101	94 – 108			121	92 – 144			104	82 – 125		
4–6	100	91 – 105	– 1.5	–3.0 – 0.0	117 †	89 – 137	–2.3	–3.8 – –0.8	103	86 – 124	– 1.4	–4.0 – 1.2
8–12	100	92 – 107	– 0.9	–2.3 – 0.5	117 †	89 – 137	–2.6	–4.1 – –1.1	101 †	77 – 123	– 2.4	–6.0 – 1.2
24–28	99	93 – 108	– 0.8	–2.2 – 0.7	116 †	87 – 136	–3.4	–5.4 – –1.5	105	80 – 123	0.0	–4.8 – 4.8
48–52	100	93 – 108	– 0.2	–1.7 – 1.4	108 †	85 – 133	–6.2	–7.7 – –4.6	101 †	79 – 120	– 2.9	–7.5 – 1.8
P-value												
Kruskal-Wallis	0.883				0.789				0.910			
Friedman	0.016				<0.001				<0.001			

NHP: Normal human plasma. IQR: Inter-quartile range. * denotes a statistically significant difference (p<0.05) compared to the control group (time <1hour) following Kruskal-Wallis One Way ANOVA on ranks. † denotes a statistically significant difference (p<0.05) compared to the control group (time <1hour) following Friedman Repeated Measures ANOVA on ranks. ‡ denotes a potentially clinically significant difference (> 10%) for the 99%-CI of the percentage change compared to the control group (time < 1hour).

Time (h)	Total protein S (n = 59)				Free protein S (n = 59)				Modified APC sensitivity ratio (n = 59)			
	Antigen (% NHP)		Change vs. <1h (%)		Antigen (% NHP)		Change vs. <1h (%)		Ratio		Change vs. <1h (%)	
	Median	(IQR)	Mean	(99%-CI)	Median	(IQR)	Mean	(99%-CI)	Median	(IQR)	Mean	(99%-CI)
<1	108	83 – 125			81	63 – 98			2.47	1.87 – 2.62		
4–6	101	80 – 119	– 3.6	–6.7 – –0.5	84	58 – 96	– 0.8	–4.1 – 2.5	2.55 †	1.98 – 2.61	2.0	0.7 – 3.2
8–12	103	83 – 118	– 3.6	–6.8 – –0.4	81	62 – 96	– 1.2	–4.4 – 2.1	2.51	1.93 – 2.61	0.9	–0.4 – 2.3
24–28	100 †	77 – 110	– 9.8 ‡	–13 – –6.5	82 †	59 – 95	– 2.7	–6.2 – –0.9	2.44	1.86 – 2.51	– 1.4	–2.7 – –0.1
48–52	92 *†	64 – 103	– 17.7 ‡	–22 – –13	80 †	59 – 92	– 4.3	–7.6 – –1.1	2.32 *†	1.83 – 2.41	– 4.3	–6.0 – –2.7
P-value												
Kruskal-Wallis	<0.001				0.955				<0.001			
Friedman	<0.001				<0.001				<0.001			

Table 5: Markers of coagulation activation.

Time (h)	Thrombin-antithrombin complex (n = 59)				D-dimer (n = 59)			
	Quantity (µg/l)		Change vs. <1h (%)		Quantity (µg/l)		Change vs. <1h (%)	
	Median	(IQR)	Mean	(99%-CI)	Median	(IQR)	Mean	(99%-CI)
<1	2.0	1.3 – 2.6			279	174 – 411		
4–6	2.0	1.6 – 2.6	13.5	–16 – 43	266	194 – 400	1.9	–3.5 – 7.3
8–12	1.9	1.6 – 2.4	0.5	–12 – 14	274	188 – 430	3.6	–1.6 – 8.8
24–28	2.2	1.8 – 2.6	15.5	–13 – 44	270	188 – 401	5.6	0.1 – 11
48–52	2.2	1.7 – 2.7	7.2	–8.5 – 23	307 †	207 – 433	9.4	3.5 – 15
P-value								
Kruskal-Wallis	0.582				0.953			
Friedman	0.066				<0.001			

* denotes a statistically significant difference (p<0.05) compared to the control group (time <1hour) following Kruskal-Wallis One Way ANOVA on ranks. † denotes a statistically significant difference (p<0.05) compared to the control group (time <1hour) following Friedman Repeated Measures ANOVA on ranks.

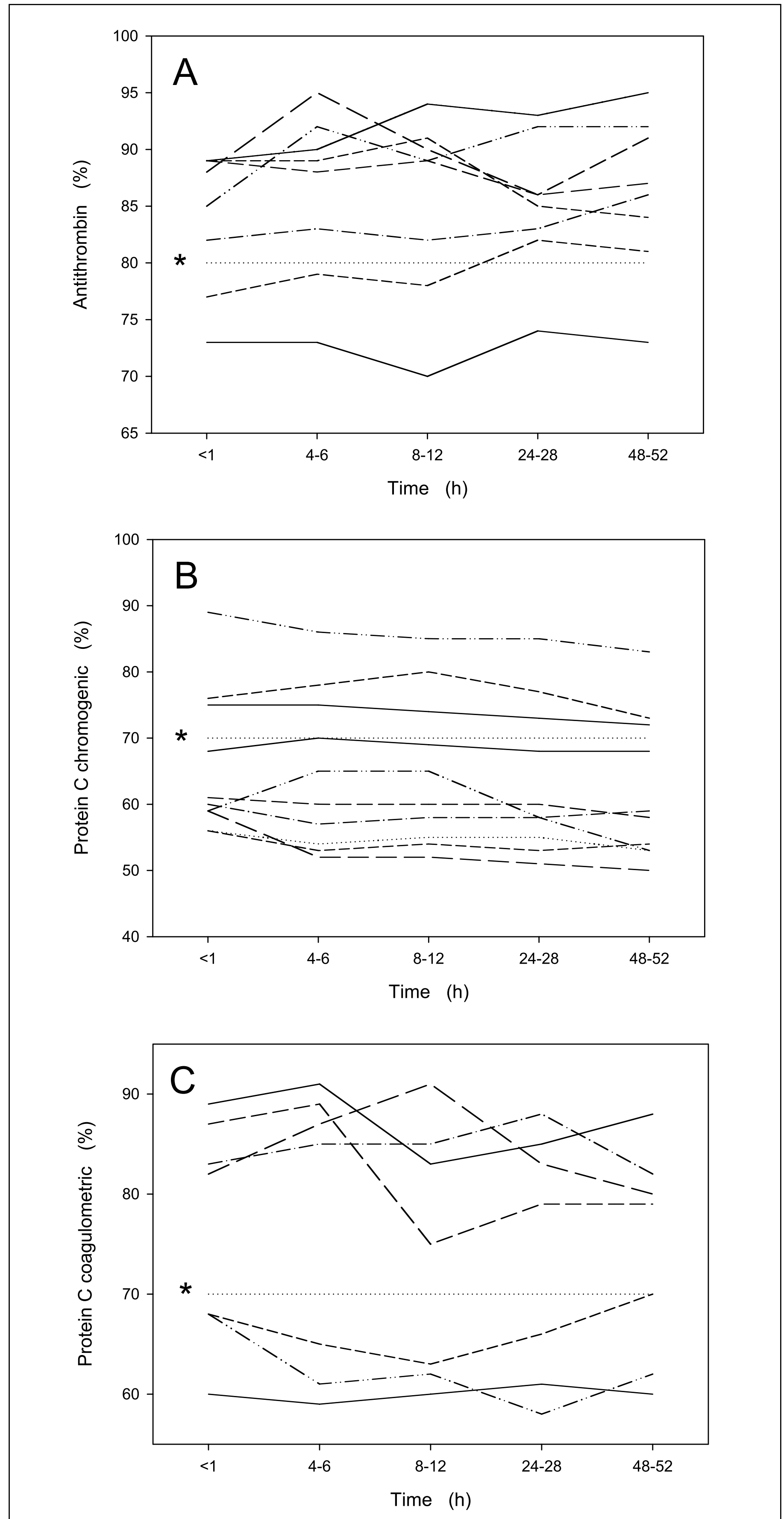

Figure 2: Natural anticoagulants. A) Antithrombin activity. Time course of all eight samples with borderline/low AT activity (70–90%). The straight dotted line (*) represents the lower limit of the normal range (80%). B) Protein C chromogenic activity. Time course of all 10 samples with borderline/low PC activity measured by chromogenic method (50–90%). The straight dotted line (*) represents the lower limit of the normal range (70%). C) Protein C coagulometric activity. Time course of all seven samples with borderline/low PC activity measured by coagulometric method (50–90%). The straight dotted line (*) represents the lower limit of the normal range (70%).

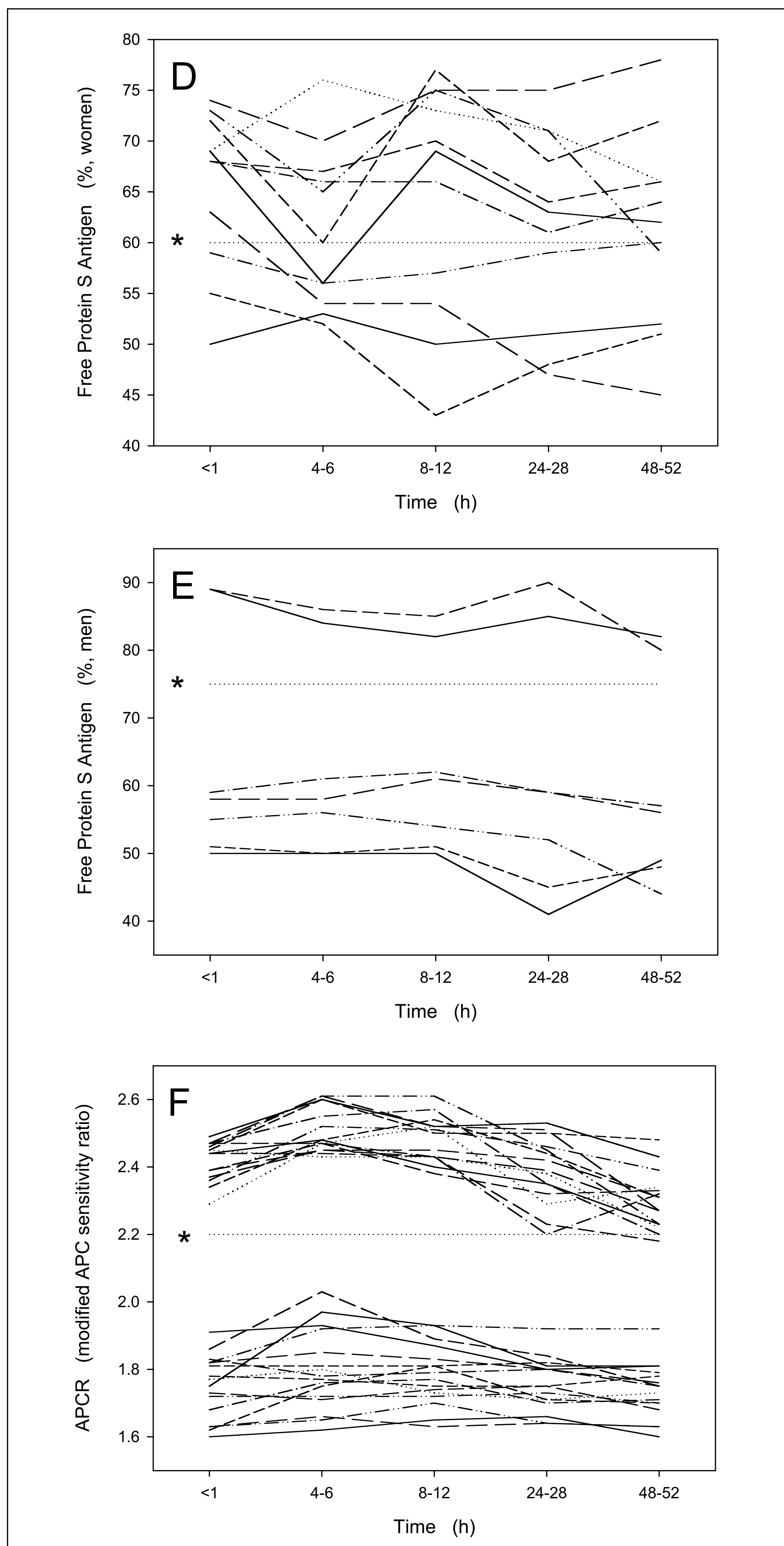

Figure 2 (continued): Natural anticoagulants. D) Free protein S antigen, women. Time course of all 11 samples with borderline/low free PS antigen values (45–75%). The straight dotted line (*) represents the lower limit of the normal range for women (60%). E) Free protein S antigen, men. Time course of all seven samples with borderline/low free PS antigen values. The straight dotted line (*) represents the lower limit of the normal range for men (75%). F) Resistance to activated protein C. Time course of the modified APC sensitivity ratio of all 16 samples with a heterozygous Factor V Leiden mutation and of all 15 samples with low-normal ratios (2.2–2.5). The straight dotted line (*) represents the lower limit of the normal range (ratio 2.2).

Our data indicate that the observed time-dependent changes of PT and aPTT are mainly due to clinically significant decreases of factors VIII:C and V:C at 24 h, and of factor VII:C at 48 h (Tables 1, 2 and 3). FV:C assessed without freezing/thawing (Table 3) appears to be more stable compared to the study samples (Table 1); however, this may be due to preanalytical coagulation activation (10). Noteworthy, all other investigated clotting factors and VWF are remarkably stable in whole blood samples transported at ambient temperature, without showing clinically significant changes up to 48–52 h after venepuncture.

We also investigated the behaviour of natural anticoagulants – AT, PC, free PS – finding clinically stable results up to 48–52 h after blood sampling. For all these parameters, the mean change (including 99%-CI) at 48–52 h was well below 10% compared to the initial measurement (Table 4). The only published study, which to our knowledge investigated a similar question (4), also demonstrated stable results for free PS antigen and observed a similar tendency for low AT values to increase with time of storage in whole blood (Fig. 2A). In contrast to the mentioned study – which reported a dramatic increase of prothrombin fragment 1+2 from 0.2 nM at day 0 to 21.2 nM at day 2 and an increase of fibrin-degradation products from 0.9 µg/ml to 12.5 µg/ml – we do not find a significant rise of activation markers (Table 5). The reason for this discrepancy is not clear. Both studies used the same citrate concentration and tube system for collecting blood samples; however, Luddington et al. do not state whether they employed a normal draw (9:1 blood to anticoagulant ratio) or a short draw (6:1). Nevertheless, our results are in line with a previous publication which demonstrated an excellent agreement between D-dimer values measured immediately after blood collection and the results obtained after storage of the respective citrated blood samples at room temperature for 24 h or transportation by train (5). D-dimer testing is useful for excluding deep vein thrombosis or pulmonary embolism in patients with a low pre-test probability (11). Moreover, it has recently been described that low D-dimers have a strong negative predictive value for recurrence after a first episode of idiopathic venous thromboembolism (12), independently of the presence of congenital thrombophilia (13). Assessment of D-dimer has, therefore, several potential clinically useful applications. Our data indicate that D-dimer testing can be safely performed in whole blood samples transported up to 48–52 h.

Since good quality venepuncture – which would particularly affect TAT and FV:C levels (10) – can not be guaranteed by the central laboratory and because borderline results could be misclassified (Fig. 2), repeat sampling and timely centrifugation of the specimen is necessary for all patients with abnormal results and for patients with low normal AT activity (see Fig. 2A and [4]). In addition, while testing for factor V Leiden with the modified APC sensitivity ratio is a cost-effective approach, for subjects with borderline results confirmation with genetic analysis of factor V gene is mandatory.

The present work indicates that in everyday practice and clinical trials involving central laboratory analysis of coagulation parameters, sample handling must be standardized, including the time between blood sampling and analysis, transportation conditions and sample freezing/thawing. Of note, the reported rapid change in factor VIII:C when PPP is left at RT (Supplemental Data Fig. 1 online at www.thrombosis-online.com) is relevant not only for patients investigated for suspected haemophilia A or von Willebrand disease type 2N (Normandy), but also for those investigated for thrombophilia. In fact, it has been showed that high FVIII:C levels strongly correlate with an increased risk of recurrent venous thromboembolism (14). For using FVIII:C levels as predictor of recurrent thromboembolism, the time delay between blood collection, PPP-preparation and sample freezing must be strictly defined.

Finally, it is important to note that the appropriate statistical method for testing whether there are differences between >2 sets of paired results (repeated measures analysis of variance) detects statistically significant differences which do not appear to be clinically relevant. For instance, PT activity at 24–28 h significantly differs from the initial values, however, with a 99%-CI for the percentage change of –6.9 – –1.6% (Table 1). Similar observations were made for FII:C, FIX:C, FX:C, FXI:C, VWF:RCo and VWF:Ag (Table 2) and for AT, PC, free PS antigen and modified APC sensitivity ratio (Table 4 and Fig. 2). In those instances, it is likely that technical variables determining the CV of each method have a greater impact on the measured results than changes of the parameter due to storage time in citrated whole blood. For example, it has been shown that the within-laboratory

Appendix: In-house between-run coefficients of variation (CV).

Assay	Normal control	Abnormal control
	% CV	% CV
PT (%)	2.16	4.57
PT (INR)	---	2.69
FII:C	4.95	5.67
FV:C	5.48	7.36
FVII:C	5.10	7.42
FX:C	5.67	7.42
aPTT	1.17	---
FVIII:C	5.60	7.68
FIX:C	5.81	6.62
FXI:C	4.83	7.73
Fibrinogen (Clauss)	5.50	---
VWF:RCo	8.36	10.45
VWF:Ag	5.91	5.79
AT	4.79	9.47
PC:coagulometric	6.67	8.56
PC:chromogenic	2.88	3.99
Modified APC sensitivity ratio	2.79	6.13
Total PS Ag	9.84	---
Free PS Ag	6.87	---
TAT-complexes	---	5.89
D-dimer	4.07	5.63

Between-runs coefficient of variation were calculated employing routine internal control results obtained over a 6-month period.

CV for AT was 7.6 % (95%-CI: 3.6–35.5%), for PC:chromogenic 6.8% (95%-CI: 3.3–24.0%), for PC:coagulometric 12.7% (95%-CI: 8.8–32.3%), and for free PS antigen 14.1% (95%CI: 6.5–79.1%) (15). We propose that for stability studies like the present one, a one way analysis of variance (or its non-parametric equivalent, the Kruskal-Wallis test) should be employed besides the technically correct analysis of variance for repeated measures. Moreover, the calculation of the mean percentage change (and its 99%-CI) from the initial value will give clinically relevant information, in particular when compared to the inhouse CV of each assay.

In conclusion, the results of our study suggest that the use of citrated whole blood samples transported by courier or first class mail at ambient temperature is an acceptable means of delivering specimens for coagulation analysis when PPP is prepared within 24–28 h after specimen collection, with the exception of the assessment of factor V and particularly factor VIII clotting activity, and the measurement of total PS antigen. Moreover, at least with the employed reagent/coagulometer combinations, the majority of the investigated analyses can be performed even 48–52 h after specimen collection without observing clinically relevant changes, which we defined as a percentage change of more than 10% from the initial measurement. Whole blood specimens, whose transportation has lasted for more than 24–28 h do not necessarily have to be refused for those tests.

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Abbreviations

APC, activated protein C; APCR, resistance to APC; aPTT, activated partial thromboplastin time; AT, antithrombin; CLSI, Clinical and Laboratory Standards Institute; CV, coefficient of variation; ELISA, enzyme-linked immunosorbent assay; FII:C, factor II coagulant activity; FV:C, factor V coagulant activity; FVII:C, factor VII coagulant activity; FIX:C, factor IX coagulant activity; FX:C, factor X coagulant activity; FXI:C, factor XI coagulant activity; INR, International Normalised Ratio; IQR, interquartile range; PC, protein C; PPP, platelet-poor plasma; PS, protein S; PT, prothrombin time; SHP, standard human plasma; TAT, thrombin-antithrombin complex; VWF:Ag, von Willebrand factor antigen; VWF:RCo, von Willebrand factor ristocetin cofactor activity.

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