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Impact of different storage times at room temperature of unspun citrated blood samples on routine coagulation tests results. Results of a bicenter study and review of the literature

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Summary

Introduction: A maximum delay between blood collection and coagulation testing of 4 hours is recommended by most guidelines. As information on optimal storage times is limited, we investigated the potential effect of different storage times of unspun tubes, that is, ≤2, 4, 6, and 8 hours, on routine coagulation test results.

Methods: Four evacuated polymer tubes containing 0.109 mol/L tri-Na citrate were drawn from 144 patients, including 39 patients on vitamin K-antagonists. Except for storage time, all tubes underwent the same preanalytical process. Prothrombin time (PT)/international normalized ratio (INR), activated partial thromboplastin time (aPTT), fibrinogen, factor V (FV), FVIII, and D-dimer were evaluated in two centers using the same technical conditions.

Results: Analytical comparison of aPTT, fibrinogen, FV, and FVIII results evaluated after prolonged storage times vs a <2-hours storage demonstrated significant difference, whereas PT/INR and D-dimer remained unchanged up to 8 hours. Mean bias between test results obtained after prolonged storage times remained below the desirable values for all studied parameters except for FVIII evaluated after 6- and 8-hours storages, but only in patients with FVIII above 100 IU/dL. Even though the corresponding bias of −5.2% and −8.5%, respectively, remained within the GEHT recommended limits of variation, its evaluation after an 8-hours storage could lead to significant underestimation of FVIII.

Conclusion: These results suggest that, in the studied technical conditions, PT/INR, aPTT, fibrinogen, FV, and D-dimer can be reliably evaluated in tubes stored unspun at room temperature for up to 8 hours after blood collection. That optimal delay should be of 6 hours for FVIII.

KEYWORDS

coagulation test, delay, hemostasis, preanalytical phase, storage

1 | INTRODUCTION

Errors within the preanalytical phase, which far exceeds those occurring during both the analytical and the postanalytical phase, account for around two-third of the total errors recorded in medical laboratories,[1] particularly for coagulation testing.[2] Accordingly, accreditation bodies are increasingly requiring laboratories to go beyond analytical quality and take responsibility for the extra-analytical, that is, pre- and

postanalytical phases where most errors were found to arise.[3] So, detailed information about preanalytical conditions has to be provided, as mentioned in the College of American Pathologists (CAP) checklist[4] or the International Organization for Standardization (ISO) 15189:2012 norm.[5] Among other items, the maximum delay between blood collection and analysis has to be defined. Up to now, the Clinical Laboratory and Standards Institute (CLSI) Guideline H21-A5[6] recommends that most coagulation parameters must be evaluated within 4 hours, except tests aimed at monitoring treatments with full-dose unfractionated heparin for whom the delay must not exceed 2 hours. Similar recommendations were made by the European Concerted Action on Thrombosis (ECAT)[7] and the Groupe Français d'Etude sur l'Hémostase et la Thrombose (GEHT).[8]

The economic pressure and the rationalization of biology have led to an increasing concentration of clinical laboratories into so-called core labs, during the last years. As a consequence, the elapsed time from specimen collection to its delivery to the laboratory has dramatically increased due to longer distances between the collection and the technical sites. In such conditions, a 4-hour delay could be a limitation in some cases, making these recommendations difficult to apply in routine practice. As the careful control of preanalytical conditions, particularly the delay between blood collection and analysis, is mandatory to avoid sample deterioration that may invalidate analysis results, longer storage times were evaluated by different groups. In most cases, there were no clinically relevant changes in test results for most parameters, particularly prothrombin time (PT)/international normalized ratio (INR), for which evaluation was found to be reliably delayed for up to 3 days with changes in test results without any significant clinical relevance.[9]

1.1 | Aim of the study

The aim of the present study was to investigate the potential impact of prolonged storage times of unspun citrated blood collection tubes on routine coagulation test results, that is, PT/INR, activated partial thromboplastin time (aPTT), fibrinogen, coagulation factors V (FV) and VIII (FVIII), and D-dimer, and so to define optimal delays between blood collection and analysis that could be used in clinical practice.

2 | PATIENTS, MATERIALS, AND METHODS

2.1 | Design of the study

For that purpose, four evacuated polymer tubes containing 0.109 mol/L tri-Na citrate (Vacutainer® Ref. 363048, Becton Dickinson, Le-Pont-de-Claix, France) were collected in two centers from outpatients. Whole blood samples were kept unspun in vertical position. Four storage times were investigated, that is, below 2, 4, 6, and 8 hours, at a temperature in the range between +18 and +25°C. Except for the storage time, all tubes underwent the same preanalytical conditions including transportation in vertical position in MoveBox (Becton Dickinson) by means of vehicle with an average distance of 15 km, and storage at a temperature between +18 and +25°C. None was transferred to the laboratory by pneumatic system. Centrifugation was performed according to the recommendations of the GEHT at 2250×*g* and +20°C for 15 minutes, and the delay between centrifugation and analysis was below 10 minutes.[8]

2.2 | Studied population

Samples were obtained from 144 outpatients (71 males and 73 females, with a mean age of 39 years, between 18 and 65). 39 patients were treated with vitamin K-antagonists (VKA), whereas 105 were not given any anticoagulant therapy. None was treated with any heparin derivative or any direct oral anticoagulant. No other specific inclusion and/or exclusion criteria were specified particularly regarding, for example, liver function or acute phase reaction. In addition, six tubes were drawn from healthy individuals to evaluate the potential impact of the storage time on the precision of the techniques. All patients/subjects gave their informed consent to participate in the study.

2.3 | Materials and Methods

At both participating centers, coagulation tests were performed using the same technical conditions regarding reagents and the ACL TOP 500 CTS analyzer. All were from the same manufacturer (Instrumentation Laboratory, IL, Bedford, MA, USA). Both centers participated to external quality assessment programs, and the z-scores were below 2.0 for all investigated parameters at the time of the study.

Prothrombin time (in seconds)/INR was evaluated using the HemosIL RecombiPlasTin 2G reagent, and aPTT (in seconds) using the HemosIL SynthASil reagent. Using the batch of reagent used in the study, the mean aPTT value was 32.0 seconds, with a normal range between 25.6 and 38.4 seconds, and that upper limit was defined as 1.20 fold the mean value. Fibrinogen (in g/L) was measured according to Clauss[10] using the HemosIL Fibrinogen C reagent. FV level (in international units per dL, IU/dL) was measured by a PT-based one-stage clotting assay using the HemosIL RecombiPlasTin 2G reagent and a specific FV-depleted plasma (HemosIL Factor V Deficient Plasma). FVIII level (in IU/dL) was evaluated by an aPTT-based one-stage clotting assay using the HemosIL SynthASil reagent and a specific FVIII-depleted plasma (HemosIL Factor VIII:C Deficient Plasma). D-dimer level (expressed in ng/mL fibrinogen equivalent unit: FEU) was measured using an automated latex-based immunoassay (HemosIL D-dimer HS 500); the cutoff level for exclusion of venous thromboembolism was 500 ng/mL.[11] As the detection limit of that D-dimer assay was 203 ng/mL on the ACL TOP analyzers, all test results below that limit were arbitrary assigned this value. When needed, assays were calibrated using lyophilized calibration plasma (HemosIL Calibration Plasma) that was calibrated against a secondary standard traceable to current World Health Organization (WHO) International Standards when available. Quality controls were performed using both normal and abnormal

lyophilized control plasmas (Normal Control, Low Abnormal Control, and Special Test Control Level 2). These lyophilized calibration and control plasmas were obtained from IL. Single lots of these reagents and plasmas were used throughout the study. Individuals methods are detailed subsequently in Table 1, which reported brand name, assay principle, within-run precision (n=30 determinations), and total precision established by evaluating normal and pathological samples in duplicate twice a day during 20 consecutive days (n=80 determinations) according to the CLSI EP05-A3 Guideline.[12] This information was given by the reagent manufacturer (IL) in the specific package inserts.

2.4 | Statistical analysis

Test results were expressed as the mean values with standard deviations (SD) in case of normally distributed data, and as the median values with their ranges when the distribution of data was not found to be normal. Time-dependent changes in test results were evaluated using repeated-measures analysis of variance (ANOVA). Analytical comparison of test results was performed using Student's *t* test for normally distributed data and otherwise using the Wilcoxon signed rank test for paired samples. *P* values below .05 were considered as significant. Moreover, the mean bias between test results obtained after different storage times was evaluated according to Bland-Altman.[13] The calculated mean bias was compared to the desirable bias derived from biologic variation that was published by Ricos et al.[14] and updated in electronic version,[15] when available, and with the GEHT recommended limits of variations (90th percentile confidence interval, CI) for hemostasis tests that were published online.[16] Statistical analysis was performed using the MedCalc software version 16.8.4 (MedCalc Software bvba, Ostend, Belgium).

3 | RESULTS

3.1 | Stability of the within-run precision with storage time

The within-run (n=6 determinations) precision, evaluated as the coefficient of variation (CV%), was determined for the studied parameters, that is, PT (in seconds), aPTT (in seconds), fibrinogen (in g/L), FV (in IU/dL), FVIII (in IU/dL), and D-dimer (in ng/mL FEU), just after blood collection (<30 minutes, T0), after a 2, a 4, a 6, an 8, a 12, and a 24-hours storage of unspun tubes between +18 and +25°C. As shown in Table 2, the CV% was found unchanged for all tested parameters when evaluated just after blood collection and after storage of the tubes for up to 24 hours, suggesting a lack of any significant deterioration of the samples within that time frame. Despite the small number of determinations (n=6), these values were found to be of the same order of magnitude that those provided by the reagent manufacturer that are reported in Table 1.

3.2 | Impact of the storage time of unspun tubes on coagulation test results

Prothrombin time, aPTT, fibrinogen, FVIII, and D-dimer test results were expressed as the median values with their ranges, as the distribution of the data was not found to be normal. INR, which was only determined in patients on VKA, fibrinogen, and FV test results, was expressed as the mean values with SD as the distribution of data was found to be normal.

Repeated-measures ANOVA demonstrated significant time-dependent changes for PT/INR, aPTT, and FVIII, whereas changes were not found to be significant for fibrinogen, FV, and D-dimer (Table 3).

TABLE 1 Specification of the analytical methods used for the measurement of prothrombin time (PT)/international normalized ratio (INR), activated partial thromboplastin time (aPTT), fibrinogen, factor V and factor VIII, and D-dimer on the ACL TOP 500 CTS analyzer: reagent brand name, assay principle, and precisions established by the manufacturer (Instrumentation Laboratory) using plasma samples both in the normal (N) and in pathological (P) ranges of concentrations; the mean levels for each parameter are given between brackets. For details, see Subjects, Materials, and Methods part

Parameters	Reagents	Principle	Within-run precision (CV%) (n=30)	Total precision (CV%)
PT (s)/INR	HemosIL RecombiPlasTin 2G	Clotting assay	N (11.9 s) : 0.8% P1 (22.0 s): 0.8% P2 (34.0 s): 0.9%	N: 2.2% P1: 3.1% P2: 3.1%
aPTT (s)	HemosIL SynthASil	Clotting assay	N (30.3 s): 1.2% P (49.3 s): 0.9%	N: 1.6% P: 2.1%
Fibrinogen (g/L)	HemosIL Fibrinogen C	Clotting assay	N (3.03 g/L): 4.5% P (1.07 g/L): 5.1%	N: 5.5% P: 6.8%
Factor V (IU/dL)	HemosIL Deficient Factor V and HemosIL RecombiPlasTin 2G	One-stage (PT-based) clotting assay	N (124.7 IU/dL): 4.0% P (43.4 IU/dL): 3.6%	N: 1.1% P: 2.4%
Factor VIII (IU/dL)	HemosIL Deficient Factor VIII and HemosIL SynthASil	One-stage (aPTT-based) clotting assay	N (100.7 IU/dL): 4.7% P (33.8 IU/dL): 5.4%	N: 3.5% P: 5.5%
D-dimer (ng/mL FEU)	HemosIL D-dimer HS 500	Latex microparticle agglutination-based assay	N (423 ng/mL): 7.2% P1 (877 ng/mL): 2.9% P2 (2469 ng/mL): 2.5%	N: 9.5% P1: 8.9% P2: 7.3%

CV, coefficient of variation; FEU, fibrinogen equivalent unit.

TABLE 2 Within-run precision (n=6 determination), evaluated as the coefficient of variation (CV%), for prothrombin time (PT, in seconds, s), activated partial thromboplastin time (aPTT, in s), fibrinogen (in g/L), factor V (FV, in IU/dL), factor VIII (FVIII, in IU/dL), and D-dimer (in ng/mL FEU). CV% was determined for the different analytes just after blood collection (<30 min, T0), after a 2-h (h), a 4, a 6, an 8, a 12, and a 24-h storage at room temperature of unspun citrated tubes

		PT (s)	aPTT (s)	Fibrinogen (g/L)	FV (IU/dL)	FVIII (IU/dL)	D-dimer (ng/mL)
Baseline test result		10.3	32.1	2.58	126.4	98.8	445.8
Coefficient of variation (CV%)	T0	1.4%	2.2%	3.9%	2.5%	4.3%	3.2%
	2 h	1.2%	1.6%	4.4%	2.8%	4.9%	3.4%
	4 h	1.6%	2.1%	2.3%	3.4%	3.5%	2.9%
	6 h	1.2%	1.7%	5.4%	1.9%	2.8%	3.1%
	8 h	1.1%	1.5%	5.1%	2.5%	5.1%	3.3%
	12 h	1.5%	1.0%	5.5%	2.4%	4.0%	3.2%
	24 h	2.2%	0.9%	5.3%	3.6%	4.8%	3.4%

3.3 | PT/INR

Repeated-measures ANOVA demonstrated significant time-dependent change for PT, and all analytical comparisons of test results obtained after a 6- and an 8-hours storage were statistically significant vs a <2-hours storage. The same was applied for INR evaluated in the 39 patients on VKA (Table 3). However, when test results obtained after prolonged storage times were compared to baseline values, according to Bland and Altman, the mean bias was found to be below the desirable values for both PT and INR (Table 4). If the range from 2.00 to 3.00 was used as the usual therapeutic range for INR, monitoring VKA therapy using collection tubes stored for up to 8 hours would have led to few cases of under- or overestimation of anticoagulation. Actually, discrepant INR values among time could only be demonstrated in the samples collected from three patients (Table 5). Two patients had borderline baseline INR (1.98), which was measured above 2.00 in at least one of the samples evaluated after a 4, 6, or 8-hours storage, and one patient had a baseline INR of 3.20, which remained above 3.00 in the samples evaluated after a 4- and a 6-hours storage, but below that limit in the sample evaluated after an 8-hours storage (2.92). However, all the differences were below the imprecision of the technique (total coefficient of variation, CV=3.1%, Table 1) and the recommended (90th percentile CI) limits of variation for the bias.[16] The individual changes in INR values measured in the tubes left unspun for 2, 4, 6, and 8 hours are reported in Figure 1.

3.4 | aPTT

Analytical comparison of aPTT test results obtained after a 4, 6, or 8-hours storage vs a <2-hours storage demonstrated statistically significant differences. However, it could be mentioned that differences between aPTT results evaluated after storages longer than 2 hours, that is, 4, 6, and 8 hours, were not statistically significant (Table 3). The mean bias, calculated according to Bland and Altman, between test results obtained after a prolonged storage vs a <2-hours storage was found to be lower than 1.2%, that is, far below the desirable values for aPTT (Table 4). While considering a prolonged aPTT as a clotting time above 38.4 seconds, which was the upper limit of the normal range using that specific lot of reagent, no case of discrepant aPTT test result could be demonstrated in the 100 untreated patients; that is, all normal baseline aPTT test results remained within the normal range after a 4, a 6, or an 8-hours storage, and the same was applied to prolonged baseline aPTT results, which remained above the upper limit of the normal range when evaluated in samples after more prolonged storage times. The only discrepancy was demonstrated in one of the 39 patients on VKA (Table 5).

3.5 | Fibrinogen

Fibrinogen test results obtained after a 4- and a 6-hours storage were significantly different from those obtained after a <2-hours storage, whereas all other comparisons failed to demonstrate any significant difference (Table 3). Comparison of test results, performed according to Bland and Altman, demonstrated mean bias below the desirable values for fibrinogen (Table 4).

3.6 | Factor V

Analytical comparison, performed using Student's *t* test for paired samples, of FV levels obtained after a 4 and a 6-hours storage vs a <2-hours storage demonstrated significant discrepancies, while the difference between test results obtained after an 8- vs a <2-hours storage was not significant (Table 3). Comparison of test results, performed according to Bland and Altman, led to mean bias below the recommended limits of variation (90th percentile) for FV (Table 4).

3.7 | Factor VIII

Analytical comparison, performed using the Wilcoxon signed rank test, of FVIII levels measured after a 4, a 6, or an 8-hours storage vs a <2-hours storage demonstrated significant discrepancies, and the same was applied to comparison of test results obtained after storage times longer than 2 hours (Table 3). Comparison, performed

TABLE 3 Potential impact of the different storage time of whole blood samples at room temperature on coagulation test results: prothrombin time (PT)/international normalized ratio (INR), activated partial thromboplastin time (aPTT), fibrinogen, factor V and factor VIII, and D-dimer

	PT (s) (n=144)	INR (n=39)	aPTT (s) (n=139)	Fibrinogen (g/L) (n=144)	Factor V (IU/dL) (n=140)	Factor VIII (IU/dL) (n=139)	D-dimer (ng/mL) (n=133)
Test results							
<2-h storage	10.9 (9.2; 63.0)	2.72±0.95	29.8 (23.0; 74.0)	3.28 (1.66; 7.10)	107.5±21.8	154.2 (61.9; 299.3)	335 (<203; 5489)
4-h storage	10.8 (9.2; 59.9)	2.72±0.92	29.8 (24.1; 74.3)	3.27 (1.58; 7.30)	110.3±22.4	149.4 (67.0; 285.4)	328 (<203; 5578)
6-h storage	10.8 (9.3; 57.3)	2.67±0.88	30.0 (24.3; 70.4)	3.27 (1.57; 7.30)	110.6±22.4	143.7 (63.4; 299.3)	361 (<203; 5639)
8-h storage	10.8 (8.9; 56.3)	2.64±0.87	29.7 (23.4; 70.6)	3.27 (1.59; 7.80)	108.7±20.7	142.7 (57.8; 289.1)	355 (<203; 5514)
Repeated-measures ANOVA (linear trend)	*P*<.0001	*P*=.0003	*P*=.032	NS (*P*=.189)	NS (*P*=.090)	*P*<.0001	NS (*P*=.191)
Analytical comparison							
4 h vs <2 h	NS (*P*=.584)**	NS (*P*=.795)*	*P*=.0004**	*P*<.0001**	*P*<.0001*	*P*<.0001**	NS (*P*=.326)**
6 h vs <2 h	*P*<.0001**	*P*=.017*	*P*=.0015**	*P*=.030**	*P*<.0001*	*P*<.0001**	NS (*P*=.121)**
8 h vs <2 h	*P*<.0001**	*P*=.0009*	*P*=.011**	NS (*P*=.131)**	NS (*P*=.106)*	*P*<.0001**	NS (*P*=.117)**
6 h vs 4 h	*P*<.0001**	*P*=.0004*	NS (*P*=.744)**	NS (*P*=.478)**	NS (*P*=.472)*	*P*=.002**	NS (*P*=.690)**
8 h vs 4 h	*P*<.0001**	*P*<.0001*	NS (*P*=.324)**	NS (*P*=.714)**	*P*=.037*	*P*<.0001**	NS (*P*=.765)**
8 h vs 6 h	*P*<.0001**	*P*=.004*	NS (*P*=.257)**	NS (*P*=.932)**	*P*=.008*	*P*<.0001**	NS (*P*=.672)**

Results were expressed as the mean values with SD in case of normally distributed data or as the median values with their ranges in case of non-normally distributed data, and analytical comparisons of test results obtained after a 4, a 6, and an 8-h storage vs T0 (<2 h) were performed using Student's *t* test (*) or Wilcoxon signed rank test (**) for paired samples, respectively. *P* values below .05 were considered to be significant. The linear trend was evaluated using repeated-measures analysis of variance (ANOVA).

TABLE 4 Mean bias, expressed in specific units and in percentage change (%), calculated according to Bland-Altman (with −1.96 +1.96 SD), between test results obtained after a 4, 6, or 8-h storage vs a <2-h storage of whole blood samples at t room temperature. Comparison with current analytical quality specifications for desirable bias derived from biologic variation as defined by Ricos et al. [14,15], and recommended limits of variation (90% confidence interval, CI) recommended by the GEHT[16]

	Calculated bias	4 h vs <2 h	6 h vs <2 h	8 h vs <2 h	Desirable bias[14,15]	Recommended limits of variation (90% CI) [16]
PT	ln seconds	0.0 (−1.0; +1.0)	−0.2 (−1.6; +1.1)	−0.4 (−2.0; +1.3)	2.0%	8.2% (PT<17 s), 14% (PT=17-23 s), 30% (PT>23 s)
	ln %	−0.2% (−5.2; +4.8)	−1.3% (−6.7; +4.1)	−1.9% (−7.9; +4.1)		
INR	ln INR	0.0 (−0.15; +0.16)	−0.04 (−0.25; +0.17)	−0.07 (−0.32; +0.18)	ND	6.0% (INR<1.60), 7.3% (INR=1.60-1.90), 10.7% (INR=1.90-3.00), 20.5% (INR>3.00)
	ln %	+0.2% (−5.1; +5.5)	−1.2% (−6.7; +4.3)	−2.3% (−8.6; +3.9)		
aPTT	ln seconds	+0.4 (−2.0; +2.7)	+0.4 (−2.4; +3.2)	+0.3 (−2.4; +3.0)	2.3%	15.6% (<33 s), 18.6% (33-50 s), 14.6% (50-69 s), 12.6% (>70 s)
	ln %	+1.1% (−6.0; +8.2)	+1.2% (−7.1; +9.4)	+0.8% (−7.4; +9.0)		
Fibrinogen	ln g/L	−0.05 (−0.35; +0.25)	−0.03 (−0.40; +0.33)	−0.03 (−0.43; +0.38)	4.8%	31.2% (<1.6 g/L), 16.3% (1.6-2.3 g/L), 11.4% (>2.3 g/L)
	in %	−1.4% (−10.5; +7.6)	−0.9% (−11.8; +9.9)	−0.8% (−12.2; +10.6)		
Factor V	ln IU/dL	+2.8 (−11.7; +17.3)	+3.2 (−11.5; +17.9)	+1.2 (−16.1; +18.5)	ND	12.5%
	ln %	+2.6% (−10.5; +15.7)	+2.9% (−10.4; +16.2)	+1.3% (−15.1; +17.7)		
Factor VIII	ln IU/dL	−5.1 (−28.2; +18.0)	−7.9 (−35.3; +19.6)	−13.3 (−40.7; +14.1)	4.9%	15.5%
	ln %	−3.2% (−17.5; +11.0)	−5.5% (−21.0; +9.9)	−8.5% (−24.1; +7.2)		
D-dimer	ln ng/mL	+10 (−70; +80)	+10 (−90; +100)	+10 (−80; +100)	8.82%[15]	ND
	in %	+1.4% (−18.5; +21.4)	+2.1% (−20.1; +24.2)	+2.3% (−24.2; +28.7)		

ND, not determined.

TABLE 5 Individual data for samples in which discrepant test results (in boldface) were found for international normalized ratio (INR), activated partial thromboplastin time (aPTT), and D-dimer when collection tubes were stored for <2, 4, 6, and 8 h at room temperature. Discrepancy was defined as a storage-induced change in test result that could have either a therapeutic or a diagnosis impact when using the INR therapeutic range=2.0-3.0, the aPTT normal range=25.6-38.4 s, and the D-dimer cutoff level=500 ng/mL

Test	<2-h storage	4-h storage	6-h storage	8-h storage
INR	3.20	3.12	3.09	**2.92**
	1.98	**2.03**	**2.02**	**2.02**
	1.98	**2.05**	1.91	1.90
aPTT (s)	37.6*	**39.1***	**38.6***	38.3*
D-dimer (ng/mL)	430	431	497	**536**
	426	**531**	491	**538**
	501	533	524	**475**
	513	540	**484**	539

*Patient on vitamin K-antagonist.

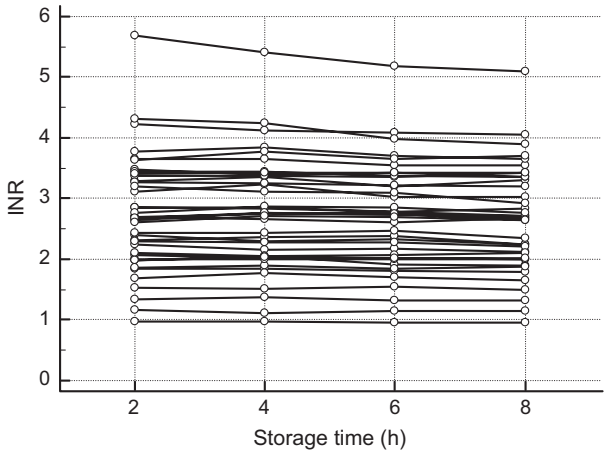

FIGURE 1 INR individual values evaluated in collection tubes obtained from 39 patients on vitamin K-antagonist that were left unspun at room temperature for <2, 4, 6, and 8 h, before being centrifuged and subsequently analyzed

according to Bland and Altman, of test results measured after prolonged storage times vs a <2-hours storage, demonstrated a time-dependent increase in the mean negative bias −3.2% after a 4-hours, −5.5% after a 6-hours, and −8.5% after an 8-hours storage (Figure 2). The mean bias was within the desirable values of 4.9% recommended by Ricos et al. [14,15] in the tubes stored for 4 hours (−3.2%), whereas it was slightly above that value after a 6-hours storage (−5.2%) and more importantly after an 8-hours storage (−8.5%). However, the latter values remained within the recommended limits of variation (90th percentile) of 15.5% recommended by the GEHT,[16] as shown in Table 4. It could be mentioned that the reported increase in the absolute value of the mean bias is also concentration dependent and only noticeable for high FVIII levels. Actually, when analyzing those 11 samples with FVIII levels below 100 IU/dL, the mean bias

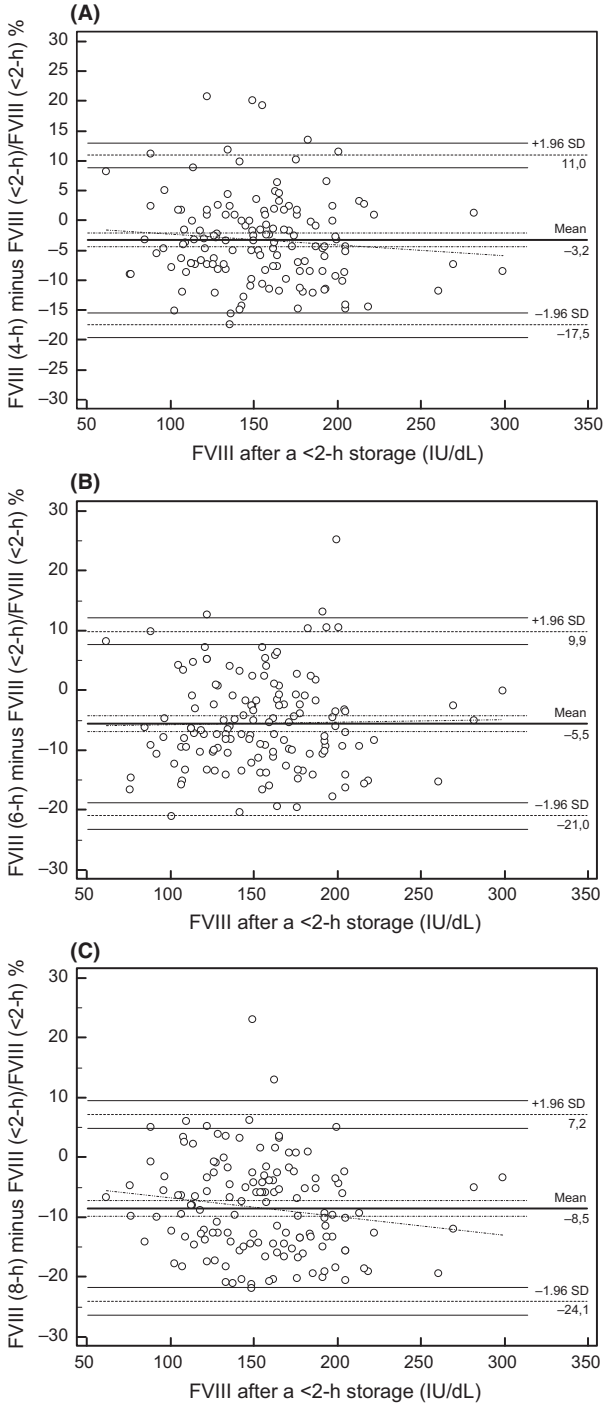

FIGURE 2 Comparison, performed according to Bland-Altman, of factor VIII (FVIII) levels (in IU/mL) measured after a 4-h (panel A), a 6-h (panel B), and an 8-h storage (panel C) vs a <2-h storage at room temperature of unspun citrated blood tubes. Results are plotted as the FVIII level measured after a <2-h storage (*x*-axis) vs the percentage changes in FVIII (*y*-axis)

between test results obtained after a 4, a 6, and an 8-hours storage and those obtained after a <2-hours storage was found very low, that is, +1.4%, −2.4%, and −2.7%, respectively (Table 6). All these values were below the desirable bias of 4.9% that was established in normal individuals.[14,15]

3.8 | D-dimer

D-dimer levels obtained after a 4, a 6, or an 8-hours storage were not statistically different from those evaluated after a <2-hours storage (Table 3). When data were analyzed according to Bland-Altman, the mean bias between test results measured after a prolonged storage vs a <2-hours storage was not clinically relevant and below the desirable bias of 8.82% (Table 4). As most of D-dimer test results were below the usually used cutoff level for VTE exclusion of 500 ng/mL, some of them being even around the lower limit of detection of the technique, we further analyzed the data set after segregating data into two groups below (n=89) and above (n=44) that threshold. As shown in Table 6, the mean bias between d-dimer levels measured after prolonged storage times was not significantly different from those measured after a 2-hours storage, with mean bias of the same order of magnitude in the two groups. Moreover, while considering 500 ng/mL as the cutoff level to exclude the diagnosis of venous thromboembolism, only four patients would have been misclassified if D-dimer was evaluated in collection tubes stored for a prolonged time vs a <2-hours storage. All these changes were within the precision of the technique around the cutoff value of 500 ng/mL (CV=9.5%) as shown in Table 5.

4 | DISCUSSION

These results suggest that PT/INR, aPTT, as well as fibrinogen, FV, FVIII, and D-dimer levels could be reliably evaluated in citrated blood tubes stored unspun at a temperature in the range from +18 to +25°C for up to 8 hours, as the impact of such prolonged storage time was either not significant or statistically significant but without any clinical relevance. If most guidelines recommend not to exceed a delay of 4 hours between blood collection and analysis,[6-8] this delay was found to be too short and not in line with real life in some cases. So there was a need to evaluate the stability of routine and even more esoteric coagulation parameters for longer times.

PT/INR is by far the most extensively studied parameter, as it is a critical test widely used in clinical practice, particularly to ensure the safety/efficacy of long-term treatments with VKA.[17] Our finding suggesting that PT/INR was stable for up to 8 hours when whole blood was stored unspun was in line with that of a previous study evaluating the potential stability of this parameter for up to 8 hours.[18] It could be noticed that the majority of previous studies evaluated the stability for longer time and concluded that PT/INR could be reliably evaluated in tubes stored up to 24 hours,[19-37] and even up to 48 hours [38-40] or 72 hours.[9] Only four studies concluded a shorter stability of PT/INR, that is, 4 hours in two studies evaluating its potential stability for up to 8 hours[41] and 24 hours,[42] and 6 hours in two studies evaluating its potential stability for up to 6 hours[43] and 24 hours.[44] Most of these studies investigated patients on VKA, even though some evaluated that parameter in healthy individuals[19,23,25,27,29,33,37,38,40,42,43] or in patients with hepatitis.[32] It could be mentioned that such a stability of PT over time was also reported in samples collected from ill dogs that were evaluated after a delay of 24[45] or 48 hours.[46]

TABLE 6 Potential impact of the different storage time of whole blood samples at room temperature on factor VIII and D-dimer test results in patients classified according to FVIII levels below and above 100 IU/dL or D-dimer levels below and above 500 ng/mL

	Factor VIII (IU/dL)		D-dimer (ng/mL)	
	<100 IU/dL (n=11)	>100 IU/dL (n=128)	<500 ng/mL (n=89)	>500 ng/mL (n=44)
Test results				
<2-h storage	88.6 (61.9; 99.7)	156.9 (100.1; 299.3)	248 (<203; 464)	828 (501; 5489)
4-h storage	87.2 (67.0; 101.8)	153.0 (87.2; 285.4)	258 (<203; 531)	851 (518; 5578)
6-h storage	82.4 (63.4; 117.2)	145.9 (79.8; 299.3)	248 (<203; 505)	836 (484; 5639)
8-h storage	84.4 (57.8; 105.3)	145.9 (84.4; 289.1)	250 (<203; 538)	820 (475; 5514)
Repeated-measures ANOVA (linear trend)	NS (*P*=.376)	*P*<.001	NS (*P*=.167)	NS (*P*=.428)
Analytical comparison				
4 h vs <2 h	NS (*P*=.898)	*P*<.0001	NS (*P*=.310)	NS (*P*=.236)
6 h vs <2 h	NS (*P*=.320)	*P*<.0001	NS (*P*=.168)	NS (*P*=.248)
8 h vs <2 h	NS (*P*=.148)	*P*<.0001	NS (*P*=.188)	NS (*P*=.178)
6 h vs 4 h	NS (*P*=.322)	*P*<.005	NS (*P*=.398)	NS (*P*=.893)
8 h vs 4 h	NS (*P*=.123)	*P*<.0001	NS (*P*=.574)	NS (*P*=.686)
8 h vs 6 h	NS (*P*=.899)	*P*<.0001	NS (*P*=.891)	NS (*P*=.786)
Mean bias (in %)				
4 h vs <2 h	+1.4 (−16.9; +19.7)	−3.4 (−17.5; +10.7)	+1.5 (−23.0; +26.1)	+0.8 (−9.0; +13.6)
6 h vs <2 h	−2.4 (−31.0; +26.2)	−5.9 (−22.2; +11.3)	+2.3 (−24.5; +29.2)	+1.0 (−8.1; +10.2)
8 h vs <2 h	−2.7 (−28.9; +28.4)	−8.7 (−24.5; +7.2)	+2.6 (−29.9; +35.1)	+1.2 (−8.8; +11.2)

Results were expressed as the median values with their ranges and analytical comparisons of test results obtained after a 4, a 6, and an 8-h storage vs T0 (<2 h) were performed using the Wilcoxon signed rank test for paired samples. *P* values below 0.05 were considered to be significant. The linear trend was evaluated using repeated-measures analysis of variance (ANOVA), and the mean bias, calculated according to Bland-Altman (with −1.96 +1.96 SD), was expressed in percentage change (%).

At this point, it could be mentioned different sources of heterogeneity among published studies. The main one was about the acceptable limits of change, as most studies considered a mean change below 10% to be acceptable, while others referred to already-published desirable bias,[14] or even considered the statistical significance of analytical comparison of test results. Another source of variation among these studies was related to the anticoagulant mixtures used in the collection tubes. The 3.2% sodium citrate solution (1 vol./9 vol.) was used in the vast majority of the studies, as it is recommended,[17] even though other anticoagulant mixtures such as the 3.8% sodium citrate,[27,29] CTAD,[37] or even sodium oxalate[34,35] solutions were also used.

Activated partial thromboplastin time, which we found to be stable for 8 hours, was less studied, and the conclusions were surprisingly more heterogeneous with a maximum stability of 2,[42] 4,[41] 6,[18,26,27,30,37,43] 8,[19,23,25,32,33] 12,[29] 24,[38] or even 48 hours.[40] Such a discrepancy could be related to difference in the studied populations, and/or reagents. However, the maximum storage time at room temperature could be as low as 2 hours[19] in patients treated with full dose of unfractionated heparin that were not included in the present study.

Fibrinogen test results were found to be unaffected by a storage up to 8 hours, with mean bias below the desirable bias,[14] in line with a previous study.[18] Moreover, other studies evaluating the stability of this parameter concluded the lack of impact of storage time on fibrinogen test results up to 24 hours[23,25,26,30,32,33] and even 48 hours.[38]

The potential impact of the storage time at room temperature on clotting factor test results was far less evaluated. We found that FV results were unchanged when evaluated after a delay of 8 hours, with a mean bias below the recommended limits of variation recently published online by the GEHT.[16] FV results were previously found to be unaffected by a storage of up to 3.5[47] or 6 hours,[43] which were the maximum evaluated times in these studies, whereas two studies concluded a stability of 24 hours when the tubes were left unspun at room temperature for up to 48 hours.[28,38]

Conflicting results were reported about FVIII, with a maximum storage time at room temperature of 2 hours,[23] below 3 hours,[48] 3.5 hours,[47] and between 4 and 6 hours,[38] when whole blood was kept unspun for up to 24, 6, 3.5, and 48 hours, respectively. However, a longer stability of 24 hours was reported for FVIII in canine blood samples.[49] We found that the mean bias between test results obtained after a 4-hours storage was below the desirable bias of 4.9% recommended by Ricos et al.,[14,15] whereas it was slightly above that limit after a 6-hours storage time (−5.5%) and more significantly after an 8-hours storage of the tubes (−8.5%). However, these values were in both cases below the GEHT recommended limit of variation.[16] The discrepancy between these two criteria is related to the evaluated population. Actually, the desirable

bias recommended by Ricos et al.[14] corresponds to intra- and inter-individual variations established in healthy individuals, whereas the GEHT recommended limits of variation[16] correspond to acceptable variations that would have no impact on patient management, and so are not limited to healthy individuals. In that connection, when statistical analysis was limited to FVIII levels below 100 IU/dL, the mean bias between test results obtained after prolonged storage times vs a <2-hours storage was far lower than those reported in the whole studied population, and even below the desirable value published by Ricos et al.[14,15] Our data suggest that FVIII could be validly evaluated in tubes stored unspun at room temperature for up to 6 hours. Even though no sample from patients with low plasma FVIII levels, such as mild hemophiliacs A or patients with von Willebrand disease, was investigated in our study, it is highly unlikely that such patients would have been misdiagnosed if FVIII was evaluated in tubes stored for prolonged time. Actually, the mean absolute value of the mean bias was found to increase with time, leading to underestimation of FVIII in stored samples, and so to underevaluate FVIII levels and possibly overdiagnose FVIII deficiency, not to misdiagnose as normal a patient with low FVIII.

D-dimer levels were found unchanged throughout the 8-hours time frame of the present study. Previous studies concluded a stability of that parameter after storage at room temperature of 24[25,30,32,33,50] and even 48 hours.[38]

Even if not evaluated in the present study, von Willebrand factor was found to be stable for up to 7 days at room temperature.[51] Concerning vitamin K-dependent factors, their levels were found unaffected by a storage time of up to 24,[21,28] and even 48 hours except for FVII (24 hours),[38] even though a lower stability of 4 hours was reported for FIX levels investigated up to 24 hours.[23] Other tests have also been investigated, such as the thrombin time that was found to be unaffected by a storage of up to 24[23,25,32,33] or even 72 hours,[40] and natural coagulation inhibitors which were found stable for up to 72 hours[40] except total protein S (24 hours).[38] A review of the literature about stability of coagulation test results in unspun whole blood samples that were stored at room temperature is summarized in Table 7.

TABLE 7 Maximal storage time at +25°C of unspun blood samples for the determination of global coagulation test, that is, prothrombin time (PT)/international normalized ratio (INR), activated partial thromboplastin time (aPTT), and thrombin time (TT), and main analyte levels, that is, fibrinogen, factor V, factor VIII, D-dimer, VWF, vitamin K-dependent factors, and coagulation inhibitors. Review of the literature: The results are expressed as the optimal storage time/maximum storage time tested by the authors

Parameters	<2 h	3-4 h	4-6 h	8 h	>8 h
PT/INR		4 h/8 h[41] 4 h/24 h[42]	6 h/6 h[43] 6 h/24 h[44]	8 h/8 h[18] 8 h/8 h This Study	24 h/24 h[19–36] 24 h/48 h[37] 48 h/48 h[38] 48 h/72 h[39, 40] 72 h/120 h[9] 24 h/48 h in dogs[45] 48 h/48 h in dogs[46]
aPTT	<2 h/24 h[42] <2 h/24 h[19]*	4 h/8 h[41]	6 h/6 h[43] 6 h/8 h[18] 6 h/24 h[26,27,30, 36] 6 h/48 h In dogs[45]	8 h/24 h[19, 23, 25, 32, 33] 8 h/8 h This Study	12 h/24 h[29] 24-28 h/48 h[38] 48 h/72 h[40] <24 h/48 h in dogs[46]
Fibrinogen			6 h/6 h[42]	8 h/8 h[18] 8 h/8 h This Study	24 h/24 h[23, 25, 26, 30, 32, 33] 48 h/48 h[37]
Factor V		3.5 h/3.5 h[47]	6 h/6 h[43]	8 h/8 h This Study	24 h/48 h[28, 38]
Factor VIII	2 h/24 h[23]	<3 h/6 h[48] 3.5 h/3.5 h[47]	4 h-6 h/48 h[38]	8 h/8 h This Study	24 h/48 h in dogs[49]
D-dimer				8 h/8 h This Study	24 h/24 h[25, 30, 32, 33, 50] 48 h/48 h[38]
VWF		3.5 h/3.5 h[47]	6 h/6 h[48]		7 days/7 days[51] 48 h/48 h In dogs[49]
VK-dep factors		3.5 h/3.5 h[47] 4 h/24 h ([23], FIX) 4 h/48 h in dogs ([45], FIX)			24 h/24 h[21] 24 h/48 h[28] 48 h/48 h ([38], except FVII: 24 h)
TT				8 h/8 h[18]	24 h/24 h[23,25,32,33] 72 h/72 h[40]
Coagulation inhibitors					24 h/24 h([27], AT) 48 h/48 h ([38], except total PS 24 h) 72 h/72 h[40]

*In patients on unfractionated heparin.

There are potential limitations to this study. Actually, the rationale behind the present study was to know until when a sample drawn in the morning could be reliably evaluated during a classic working day. For that purpose, and for logistical reasons, we decided to evaluate storage times up to 8 hours, and not to 24 hours, as it was the case in some of the previous publications. In addition, except for PT/INR in patients on VKA, baseline test results were within the normal range for most of the studied parameters in our patients' population. Particularly, FVIII levels were found above 100 IU/mL in most of the studied patients, only 11 of them having levels below that level. As the lowest FVIII level was 61.9 IU/mL, our conclusion must be extrapolated with caution to patients with FVIII levels below that value. However, as the trend was found to be toward decreasing FVIII test results with increasing storage time, the misdiagnosis as normal of a patient with mild hemophilia A or moderate von Willebrand disease is highly unlikely.

In conclusion, our study confirms that a prolonged storage time up to 8 hours could induce statistically significant changes for some routine coagulation test results, but that these changes remained within the desirable limits of variation, and had no clinical relevance. Accordingly, the measurement of PT/INR, aPTT, fibrinogen, FV, and D-dimer, using the investigated reagents/analyzer, could be reliably evaluated in citrated blood samples stored unspun between +18 and +25°C for up to 8 hours after blood collection. That optimal delay should be 6 hours for FVIII. However, the conclusions might be different if a laboratory encounters different conditions and/or analytical systems.

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