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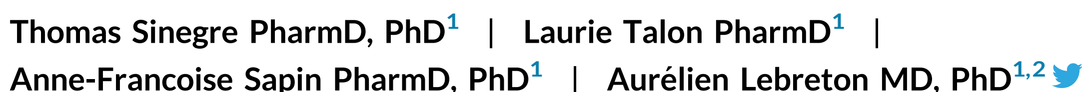
BRIEF REPORT

Effect of tube filling on plasma freezing for coagulation testing



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Abstract

Background: Storage of frozen plasma samples for hemostasis testing is a key step to obtain reliable results. Variables that can affect the quality of plasma during storage include the cryotube type and volume and the tube filling level that conditions the residual air volume. To date, there are only few data on which to base recommendations.

Objectives: The aim of this study was to investigate the influence of the tube filling volume (20%, 40%, and 80%) of 2-mL microtubes on frozen plasma for a large panel of hemostasis assays.

Methods: For this study, 85 subjects were included, and blood samples were collected from them by venipuncture. After double centrifugation, each sample was aliquoted in 3 2-mL microtubes with different volumes (0.4, 0.8, and 1.6 mL) and stored at −80 °C. At the end of the frozen storage period (3 months ± 1 week), all aliquots from the sample were tested in the same analytical series for a large panel of hemostasis analyses.

Results: Compared with completely filled microtubes (1.6/2 mL), storing frozen plasma in smaller volumes (0.4/2 mL) significantly decreased prothrombin time and activated partial thromboplastin time. Conversely, factor II, V, VII, and X levels were increased. Antithrombin, Russell's viper venom time, and anti-Xa activity in patients treated with heparin were also increased.

Conclusion: To store plasma at −80 °C for hemostasis analysis, samples should be frozen in small-volume microtubes (<2 mL) with screw caps that are filled to 80% of their volume.

KEYWORDS
coagulation testing, freezing, hemostasis, plasma, preanalytical phase

Essentials

- The preanalytical phase is a key step in clinical laboratories, especially for coagulation tests.
- Sample freezing conditions (tube type and plasma volume) may affect results.
- The use of microtubes adapted to the frozen volume is preferable to 5-mL tubes.
- An optimal filling of 80% of the microtubes is recommended for storage at −80 °C.

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1 | INTRODUCTION

The preanalytical phase is a key step in clinical laboratories, particularly for coagulation assays. It can represent more than half of the errors at the origin of inaccurate results that lead to degraded care or errors and costs for the institution [1]. Among the preanalytical variables, sample storage is an important parameter. The stability of blood samples (as whole blood or as plasma) for hemostasis analyses has been widely evaluated, and scientific societies have already provided several recommendations [2,3]. Conventional coagulation parameters are generally analyzed using fresh plasma immediately after centrifugation. However, owing to the centralization of laboratories and the need for specialized analyses, controls, or clinical studies, plasma samples may be frozen and stored for later analysis. The stability of frozen samples has been extensively studied and may vary with the storage temperature (−20 °C or −80 °C) or the thawing mode [4,5]. However, other variables that may affect the sample conservation quality also deserve to be better investigated—for instance, the cryotube type and the filling volume. The French Haemostasis Society recommends that for long storage durations (>3 months), cryotubes and frozen plasma volume should be matched, allowing as little residual air as possible, and screw caps should be preferred [3]. However, these recommendations are based only on the study by Woodhams et al. [6], who compared 2 tube types: 5-mL plastic tubes with push-on stopper and 1.5-mL microtubes with screw stopper. Moreover, because the tube filling volume was similar (3/5 mL [60%] and 1/1.5 mL [66%]) [6], this study focused more on the tube type rather than on the filling volume.

Therefore, the aim of this study was to evaluate the influence of the microtube filling volume (20%, 40%, and 80%), and the cryotube type (2-mL microtube vs 5-mL tube) on frozen plasma storage for a large panel of hemostasis assays.

2 | PATIENTS AND METHODS

2.1 | Blood sampling and plasma preparation

This prospective study was performed at Clermont-Ferrand University Hospital, France (ISO 15189–certified laboratory), between January 2021 and December 2021. The study was approved by the local ethics committee, and all experiments were performed in accordance with French laws (CPP Sud-Est VI, ref. AU765). Informed consents were obtained from all participants of this study. The first 90 patients with spare blood samples and for whom hemostasis analyses were prescribed were included. Finally, only 85 subjects were included in the study, and 5 were excluded because of poor sample quality (hemolysis and latescence) or too little plasma available. Among the 85 included patients (55% men; mean age [minimum to maximum], 51 years [30-74 years]), 65 were not receiving any anticoagulant treatment and 7 were receiving unfractionated heparin and 13 vitamin K antagonists at sample collection. Blood was collected by venipuncture in 0.109-M citrate tubes (Beckton Dickinson) after discarding the first few milliliters. Platelet-poor plasma was prepared by double centrifugation (2500*g* for 15 minutes at 21 °C), with an intermediate step of plasma decantation. Samples were immediately stored at −80 °C in 2-mL microtubes (0.4-, 0.8-, and 1.6-mL filling volume) or in a 5-mL tube (1-mL filling volume) until testing (3 months ± 1 week). Before analyses, frozen plasma samples were thawed in a water bath at 37 °C for 5 minutes.

2.2 | Coagulation assays

At the end of the frozen storage period (3 months ± 1 week), all aliquots of the sample from the same subject were tested in the same analytical series. For each of the 85 samples included in the study, assays were selected from the following test: prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen (Clauss method), factor (F)II, FV, FVII, FX, FVIII, FIX, FXI, FXII, physiological inhibitors, activities of antithrombin, protein C and protein S, von Willebrand factor (activity and antigen), dilute Russell's viper venom time (DRVVT) screen and confirm assays, and anti-Xa activity. All tests were performed on a STAR Max coagulometer (Stago) with dedicated reagents and according to the manufacturer's instructions, except for the von Willebrand factor activity-ristocetin cofactor test performed on an AcuStar instrument (Werfen).

2.3 | Statistical analysis

Statistical analyses were performed with the Prism software (GraphPad). Data were presented as median (Quartile1-3). Differences between frozen plasma volumes were analyzed with analysis of variance (ANOVA) or the Friedman test, followed by the appropriate multiple-comparison post hoc (Tukey-Kramer or Dunn) test. The mean bias was calculated using the Bland-Altman method between the test results obtained with the 2 tube types.

3 | RESULTS AND DISCUSSION

3.1 | Effect of tube filling volume

The results of the comparison of the 3 different filling volumes (1.6, 0.8, and 0.4 mL) for the 2-mL microtubes are shown in the Table. Compared with the fully filled microtube (1.6/2 mL), using plasma stored in a small volume (0.4/2 mL) led to shorter coagulation times with a drop in PT from 14.1 seconds (13.1-16.1 seconds) to 13.6 seconds (12.8-15.3 seconds) ($P < .001$) and a drop in aPTT from 36.0 seconds (32.4-43.4 seconds) to 34.9 seconds (31.8-42.4 seconds) ($P < .001$). The mean biases were 3.3% for PT and 4.0% for aPTT. These variations are compatible with an increase in the levels of all extrinsic pathway factors (FII, FV, FVII, and FX). Notably, FV increased from 104% (86%-127%) to 111% (9%8-128%) ($P < .01$), with a bias of 7.6%. Fibrinogen also increased from 3.7 g/L (2.9-5.2 g/L) to 4.1 g/L (3.4-5.9

TABLE Effect of the cryotube filling level on hemostasis parameters

Assay	Number	Frozen plasma 1.6/2 mL (80%)	Frozen plasma 0.8/2 mL (40%)	Frozen plasma 0.4/2 mL (20%)
PT (s)	*n* = 65	14.1 (13.1-16.1)	13.9 (13.3-15.8)	13.6 (12.8-15.3)[a,b]
aPTT (s)	*n* = 65	36.0 (32.4-43.4)	35.8 (32.5-45.6)	34.9 (31.8-42.4)[a,b]
Fibrinogen (g/L)	*n* = 63	3.7 (2.9-5.2)	3.6 (3.0-5.5)	4.1 (3.4-5.9)[a,b]
Factor II (%)	*n* = 34	94 (83-109)	95 (79-109)	101 (84-116)[c,d]
Factor V (%)	*n* = 33	104 (86-127)	107 (90-126)	111 (98-128)[c,e]
Factor VII (%)	*n* = 31	116 (77-130)	114 (76-138)	114 (83-149)[a,b]
Factor X (%)	*n* = 34	108 (85-120)	104 (78-124)	103 (88-133)[c,e]
Factor VIII (%)	*n* = 10	154 (123-192)	164 (131-208)	183 (127-222)
Factor IX (%)	*n* = 10	131 (122-140)	134 (117-137)	135 (126-141)
Factor XI (%)	*n* = 10	111 (101-123)	113 (102-136)	114 (110-136)
Factor XII (%)	*n* = 10	114 (99-127)	113 (99-124)	120 (109-129)
vWF antigen (%)	*n* = 10	128 (119-1`63)	142 (113-171)	148 (116-211)
vWF Rco (%)	*n* = 5	111 (104-127)	116 (102-133)	115 (102-158)
Protein C (%)	*n* = 10	141 (108-155)	141 (112-156)	129 (108-148)
Protein S (%)	*n* = 10	99 (75-105)	97 (84-102)	100 (87.25-108)
Antithrombin (%)	*n* = 33	103 (85-107)	100 (91-107)	108 (98-113)[b,c]
DRVVT screening (%)	*n* = 10	37.0 (33.9-38.2)	37.2 (34.4-38.4)	37.7 (34.5-39.0)[e,f]
DRVVT confirm (%)	*n* = 10	34.4 (32.6-35.1)	34.2 (32.3-34.7)	34.4 (32.6-35.0)
Anti-Xa activity (U/mL)	*n* = 7	0.21 (0.12-0.73)	0.19 (0.13-0.70)	0.23 (0.12-0.80)[f]

aPTT, activated partial thromboplastin time; DRVVT, dilute Russell's viper venom time; PT, prothrombin time; Rco, ristocetin cofactor; vWF, von Willebrand factor.
[a]$P < .001$, compared with frozen plasma in 80% filled tubes (1.6/2 mL).
[b]$P < .001$, compared with frozen plasma in 40% filled microtubes (0.8/2 mL).
[c]$P < .01$, compared with frozen plasma in 80% filled tubes (1.6/2 mL).
[d]$P > .05$, compared with frozen plasma in 40% filled microtubes (0.8/2 mL).
[e]$P < .01$, compared with frozen plasma in 40% filled microtubes (0.8/2 mL).
[f]$P > .05$, compared with frozen plasma in 80% filled tubes (1.6/2 mL).

g/L) ($P < .001$), with a significant bias of 11.1%. A tendency (not significant) toward increase was observed for the intrinsic pathway factors (FVIII, FIX, FXI, and FXII) when using the 0.4/2-mL samples. Similarly, the levels of von Willebrand factor antigen and ristocetin cofactor tended to increase (not significant) with biases of 6.7% and 8.8%, respectively. Physiological inhibitors showed distinct profiles. Protein C and protein S remained stable regardless of the filling volume, whereas antithrombin levels increased from 103% (85%-107%) to 108% (98%-113%) ($P < .01$), with a bias of 6.4%. The DRVVT screening assay increased from 37.0 seconds (33.9-38.2 seconds) to 37.7 seconds (34.5-39.0 seconds) ($P < .05$), with a bias of 2.3%. Conversely, the DRVVT confirm assay results were comparable with both high and low volumes (bias of 0.0%). Anti-Xa activity levels in patients treated with unfractionated heparin (n = 7) increased from 0.21 U/mL (0.12-0.73 U/mL) to 0.23 U/mL (0.12-0.80 U/mL), with a bias of 12.7%. Of note, the results obtained using the plasma stored in the intermediate filling tubes (40%; 0.8/2 mL) were comparable with those obtained with the completely filled microtubes.

3.2 | Effect of tube type

The results of the comparison of 2 tube types (2 ml vs 5 ml) were obtained using the same filling level (20%) that corresponds to a volume frequently found in the laboratory (1 mL in 5 mL) (Figure). Compared with the 2-mL microtubes, using 5-mL tubes for freezing did not seem to influence the PT measurements, whereas aPTT increased from 34.9 seconds (31.8-42.4 seconds) to 35.2 seconds (32.5-45.2 seconds) ($P < .001$), with a mean bias of 0.8 seconds. Conversely, fibrinogen decreased from 4.1 g/L (3.4-5.9 g/L) to 3.8 g/L (3.0-5.0 g/L) ($P < .001$), with a bias of 0.4 g/L. Extrinsic pathway factors tended to decrease with a bias of ~4%. The most important biases were found for high values of these parameters. This can be explained by additional plasma dilution procedures. The difference was significant for FVII (114% [83%-149%] vs 108% [85%-124%]; $P < .01$) and for FX (103% [88-133] vs 98% [85-124]; $P < .001$). Conversely, the levels of the intrinsic pathway factors were not influenced by the tube type. Physiological inhibitors showed distinct profiles. Antithrombin

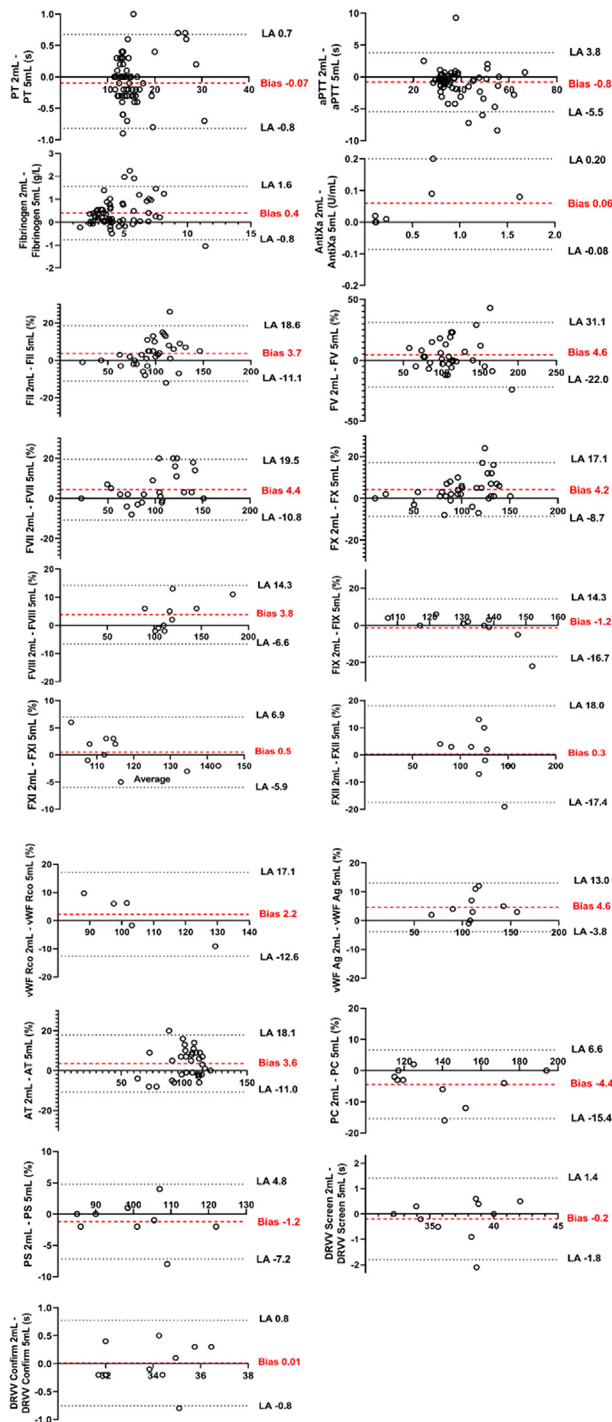

FIGURE Bland-Altman plots showing the results of the hemostasis analyses performed using samples frozen in 2-mL microtubes and 5-mL tubes. Ag, antigen; aPTT, activated partial thromboplastin time; AT, antithrombin; DRVV, dilute Russell's viper venom; F, factor; LA, limit of agreement; PC, protein C; PS, protein S, PT, prothrombin time; Rco, ristocetin cofactor; vWF, von Willebrand factor.

activity decreased from 108% (98%-113%) to 102% (94%-111%) ($P < .01$), protein C activity increased from 130% (117%-144%) to 134% (119%-156%) ($P < .05$), and protein S activity remained unchanged. The DRVVT screening and confirm assay results were also comparable between cryotube types.

Several hypotheses can explain these results. Trondsetås et al. [7] have shown that exposure to dry ice leads to an increase in pH probably because of carbon dioxide diffusion from the sample to ambient air. This increase in pH, also described during prolonged storage without a cap, leads to a lengthening of coagulation times. This gas exchange seems to depend on the sample surface in contact with air and can be minimized using small-caliber tubes [8]. Alternatively, the concentration of coagulation proteins could be increased by the evaporation process that might occur during thawing in a water bath at 37 °C, when the tubes are subsequently uncapped. In addition to pH variations and evaporation, the buffer capacity of the reagents and the analytical methodologies used, including plasma dilutions [8], could also explain these results. A study to investigate the underlying mechanisms deserves to be performed.

Our results showed that the microtube filling volume and the dead space in the tube are variables to be considered when freezing plasma dedicated to hemostasis assays, especially conventional tests (eg, PT, aPTT, FII, FV, FVII, FX, antithrombin, DRVVT assays, and anti-Xa activity). Although some differences were significant, the effect of this preanalytical variable seems minor and probably is not clinically significant. Yet, these variations might become relevant in the case of a severe deficiency. It is important to take into account this information for the sample overall management, and a minimum filling volume of 40% seems appropriate. Moreover, because of the multiplicity and specificity of automaton/reagent systems in hemostasis, these results should be confirmed with other analysis platforms. In our study, we evaluated plasma samples after 3 months of storage; however, some hemostasis parameters can be tested in plasma stored for up to 24 months [2,9]. More studies are needed to confirm our results and extend the storage periods under study. Moreover, our findings concern plasma stored at −80 °C. However, some hemostasis parameters can also be assessed in plasma stored at −20 °C but with reduced stability. Therefore, the filling volume effect should also be evaluated at −20 °C because these conditions may modify evaporation [10,11].

4 | CONCLUSIONS

On the basis of our results, we propose that, to store plasma at −80 °C for up to 3 months before hemostasis analysis, small-volume microtubes (<2 mL) with screw caps should be used, and they should be filled to 80% of their volume (or at least 40%).

AUTHOR CONTRIBUTIONS

T.S. designed the study, performed the statistical analysis, and wrote the initial draft of the manuscript. A.L., L.T., and A.-F.S. critically reviewed the draft.

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ETHICS STATEMENT

The study was approved by the local ethics committee, and all experiments were performed in accordance with French laws (CPP Sud-Est VI, ref. AU765). Informed consents were obtained from all participants of this study.

RELATIONSHIP DISCLOSURE

T.S. is a consultant for Stago for protein S and PDF analysis. A.L. is on the advisory board for Sobi, Takeda, Roche, LFB, Octapharma, Pfizer, and Bayer; is a speaker (at symposiums) for Sobi, Octapharma, and Takeda; received support for congress, including travel, from Sobi, Novonordisk Roche, and Octapharma; and received reagents for studies from Stago, Horiba, Werfen, and Siemens. There are no other competing interests to disclose.

TWITTER

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