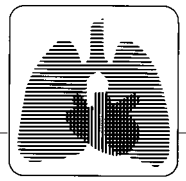

# laboratory and animal investigation

# Impact of Evacuated Collection Tube Fill Volume and Mixing on Routine Coagulation Testing Using 2.5-mL (Pediatric) Tubes*

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***Background:*** **Anecdotal observations by pharmacists monitoring anticoagulated patients indicate that blood samples collected in 2.7-mL (pediatric) evacuated tubes frequently produced falsely elevated international normalized ratio (INR) results.**
***Objective:*** **To evaluate the impact of various preanalytical variables (fill volume, sample mixing, and elapsed time between sample collection and mixing) on INR test results using pediatric collection tubes in healthy volunteers and patients receiving warfarin anticoagulation therapy. Fifteen patients receiving warfarin and the 15 healthy volunteers participated in each study arm.**
***Methods:*** **Multiple blood samples for coagulation testing were obtained from study subjects in full-draw pediatric collection tubes made of siliconized glass. The impact of sample mixing was evaluated by randomly varying the number of times each tube (five tubes total) was inverted following sample collection between one and five. The impact of timely sample mixing was evaluated by randomly varying the elapsed time between sample collection and mixing between 0 min and 4 min in each of five samples. The impact of incomplete collection tube filling was evaluated by randomly varying the volume of six tubes between 50% and 100%. Duplicate coagulation assays were performed on each sample by a centralized hematology laboratory, and the average result was reported.**
***Results:*** **Statistical analysis revealed that neither sample mixing nor the elapsed time between sample collection and mixing had a statistically significant effect on INR test results. For patients receiving warfarin, tube fill volume had a statistically significant effect on the reported INR results ($p < 0.001$). The mean (± SD) INR derived from sample tubes filled 100% was 3.2 ± 1.2, compared to 9.9 ± 4.2 for tubes filled only 50% full ($p < 0.01$). Statistically significant INR elevations became apparent for sample tube fill volumes of < 90%.**
***Conclusion:*** **Pediatric blood collection tubes should be filled at least 90% full to ensure accurate INR test results. Anticoagulation therapy providers should routinely inquire about the type of collection tube used (adult vs pediatric) and the adequacy of sample collection volume before deriving therapeutic plans in asymptomatic excessively anticoagulated patients.**

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**Key words:** anticoagulants; blood specimen collection; international normalized ratio; prothrombin time; warfarin

**Abbreviations:** CPAS = Clinical Pharmacy Anticoagulation Service; INR = international normalized ratio; PT = prothrombin time

Patients receiving oral anticoagulation therapy with warfarin sodium require frequent international normalized ratio (INR) monitoring to ensure adequate anticoagulant response, and to reduce the risk of bleeding complications due to excessive anticoagulation. The INR is derived from the prothrom-

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bin time (PT), which is one of the most commonly performed clinical laboratory procedures.[1] Numerous preanalytical variables associated with the collection, transportation, and processing of blood samples may affect the results of routine coagulation assays.[2] Preanalytical variables related to the evacuated tube used to collect blood samples include the concentration of sodium citrate (3.2% vs 3.8%), sample fill volume, and the mixing of whole blood and liquid anticoagulant (sodium citrate) in the tube.

Pharmacists monitoring anticoagulated patients in our Clinical Pharmacy Anticoagulation Service (CPAS) anecdotally noted that falsely elevated INR results occurred more frequently when blood samples for coagulation testing were drawn in 2.7-mL (pediatric) evacuated tubes. Spuriously elevated PT values have been reported in underfilled evacuated tubes.[1,2] The National Committee for Clinical Laboratory Standards recommends that coagulation samples be rejected if the evacuated tube contains $< 90\%$ of the expected fill volume.[3] However, research using 5-mL (adult), full-draw evacuated tubes made of siliconized glass has demonstrated that accurate INR results are obtained with a minimum fill volume of 70% and 60% evacuated tubes containing 3.8% and 3.2% sodium citrate, respectively.[2] Similar studies to determine the minimum necessary fill volume for pediatric evacuated tubes are not available.

National Committee for Clinical Laboratory Standards guidelines[3] further recommend that evacuated tubes should be immediately inverted three to four times to ensure adequate mixing of whole blood with liquid anticoagulant. No published studies have evaluated the effect number of inversions or the time from sample collection to inversion has on the accuracy of INR results.

In an attempt to explain the mechanism underlying the aforementioned clinical observation, we conducted a randomized controlled trial evaluating the effect of three preanalytical variables, namely evacuated tube fill volume, number of tube inversions, and the time from sample collection to inversion, using pediatric evacuated collection tubes.

## Materials and Methods

### *Setting*

The study was conducted in the Kaiser Permanente Colorado Region, a nonprofit, group-model heath maintenance organization. The CPAS at Kaiser Permanente Colorado Region provides comprehensive services for all patients requiring anticoagulation therapy in the Denver-Boulder area. The CPAS currently monitors approximately 5,900 patients. The physicians of the Colorado Permanente Medical Group refer patients requiring anticoagulation therapy to the CPAS. Working in conjunction with the referring physician, CPAS clinical pharmacy staff initiates anticoagulation therapies, orders relevant laboratory tests, adjusts anticoagulation medications as necessary, and refills anticoagulation medications. CPAS pharmacy staff members are also responsible for providing patient education, drug interaction evaluation and management, compliance assessment, plans for interrupting anticoagulation therapy during invasive procedures, and facilitating management of anticoagulation therapy complications. The service is able to provide care for ambulatory, homebound, and nursing home patients, as nearly all CPAS patient-care activities are conducted via telephone. Each clinical pharmacist is responsible for 300 to 500 patients and works with the same patients over time.

Venous blood samples for PTs are analyzed by a centralized hematology laboratory. INR values are derived from prothrombin times measured by the Sysmex CA 6000 instrument using Innovin thromboplastin (Dade Behring; Newark, DE). The international sensitivity index value for the thromboplastin used during this investigation was 0.9.

### *Study Subjects*

Patients receiving oral anticoagulation therapy with warfarin were recruited from among CPAS patients $\geq$ 18 years old visiting one of our medical office laboratories for routine INR testing. Patients receiving concurrent therapy with low molecular weight or unfractionated heparin or with a history of lupus anticoagulant were excluded due to potential for false elevations in INR results. Eleven of the 45 patients receiving warfarin therapy were also receiving aspirin. Healthy volunteers $\geq$ 18 years old were recruited from among health-plan staff at the same medical office. All patients signed informed consent prior to participating in the study. The study protocol was conducted in accordance with the recommendations found in the Helsinki Declaration of 1975, and was reviewed and approved by the Kaiser Foundation Research Institute Biomedical Institutional Review Board.

### *Intervention*

Blood was collected via venipuncture into full-draw, 3.2% citrate, 2.7-mL (pediatric) tubes made of siliconized glass (Vacutainer; Becton Dickinson; Franklin Lakes, NJ). All samples were centrifuged immediately and the supernatant divided in aliquots for later coagulation testing. Samples were transported to a central hematology laboratory where the aliquots were frozen ($-20$°C). Routine coagulation tests were performed within 2 weeks of sample collection. Duplicate PT assays were performed on each sample. The INR result was calculated using the PT according to usual practice at our institution. Results were logged onto a data sheet that included identification number, laboratory accession number, and duplicate PT results for each sample. The technologist performing the analysis initialed each entry.

The study was performed using three protocols. Fifteen patients receiving warfarin and 15 healthy volunteers participated in each protocol. The first protocol was designed to evaluate the effect of blood sample mixing with liquid anticoagulant in the evacuated tube (mixing protocol). For each study subject, five pediatric evacuated tubes were completely filled (100%) and immediately inverted one, two, three, four, or five times. The number of inversions sequence for each study subject was determined randomly.

The second protocol was designed to evaluate the effect of elapsed time between blood sample collection and mixing with liquid anticoagulant (time to mixing protocol). For each study subject, five pediatric tubes were 100% filled and inverted five times. The samples were inverted at 0, 1, 2, 3, or 4 min following collection in a randomized order.

The third protocol was designed to evaluate the effect of evacuated tube fill volume (fill volume protocol). For each study subject, six pediatric tubes were collected and immediately inverted five times. The evacuated tubes were filled 100%, 90%, 80%, 70%, 60%, or 50% full. The fill volume sequence for each study subject was determined randomly.

### *Statistical Analysis*

Results were analyzed using Friedman analysis of variance. Two-tailed p values $< 0.05$ were considered statistically significant. *Post hoc* tests with Bonferroni correction were employed to delineate actual differences between groups when analysis of variance results were statistically significant.

## Results

A total of 45 anticoagulated patients and 30 healthy volunteers participated in the study. Fifteen healthy volunteers participated in two of the study protocols. Study subject demographic data are summarized in Table 1.

**Table 1—*Study Subject Demographic Data***

| Variables | Data |
|---|---|
| Mixing protocol | |
| Healthy volunteers (n = 15) | |
| Mean age, yr (SD) | 40.4 (8.8) |
| Female gender, No. (%) | 9 (60.0) |
| Patients receiving warfarin (n = 15) | |
| Mean age, yr (SD) | 70.1 (8.9) |
| Female gender, No. (%) | 7 (46.7) |
| Primary indication for warfarin therapy, No. (%) | |
| Venous thromboembolism | 2 (13.3) |
| Atrial fibrillation/flutter | 9 (60.0) |
| Arterial thromboembolism | 1 (6.7) |
| Stroke/cerebrovascular accident | 2 (13.3) |
| Heart valve prosthesis | 1 (6.7) |
| Time to mixing protocol | |
| Healthy volunteers (n = 15) | |
| Mean age, yr (SD) | 43.7 (11.2) |
| Female gender, No. (%) | 6 (40.0) |
| Patients receiving warfarin (n = 15) | |
| Age in years, mean (SD) | 61.8 (7.8) |
| Female gender, No. (%) | 4 (26.7) |
| Primary indication for warfarin therapy, No. (%) | |
| Venous thromboembolism | 4 (26.7) |
| Atrial fibrillation/flutter | 7 (46.7) |
| Stroke/cerebrovascular accident | 2 (13.3) |
| Heart valve prosthesis | 2 (13.3) |
| Fill volume protocol | |
| Healthy volunteers (n = 15) | |
| Mean age, yr (SD) | 43.5 (9.2) |
| Female gender, No. (%) | 9 (60.0) |
| Patients receiving warfarin (n = 15) | |
| Mean age, yr (SD) | 69.5 (13.0) |
| Female gender, No. (%) | 7 (46.7) |
| Primary indication for warfarin therapy, No. (%) | |
| Venous thromboembolism | 3 (20.0) |
| Atrial fibrillation/flutter | 9 (60.0) |
| Arterial thromboembolism | 1 (6.7) |
| Stroke/cerebrovascular accident | 2 (13.3) |

**Table 2—*Mixing Protocol Results***

| Study Group/No. of Inversions | Mean INR (SD) | p Value |
|---|---|---|
| Healthy volunteers (n = 15) | | |
| 5 | 1.1 (0.05) | 0.77 |
| 4 | 1.1 (0.05) | |
| 3 | 1.1 (0.05) | |
| 2 | 1.1 (0.05) | |
| 1 | 1.1 (0.05) | |
| Warfarin patients (n = 15) | | |
| 5 | 2.8 (1.2) | 0.66 |
| 4 | 2.7 (1.3) | |
| 3 | 2.7 (1.2) | |
| 2 | 2.8 (1.4) | |
| 1 | 2.8 (1.3) | |

### *Mixing Protocol*

Statistical analysis revealed no significant differences in the mean INRs across the five different number of inversions in normal healthy volunteers or patients receiving warfarin (Table 2; $p = 0.77$ and $p = 0.66$, respectively).

### *Time to Mixing Protocol*

Statistical analysis revealed no significant differences in the mean INRs across the five different times to evacuated tube inversion in normal healthy volunteers or patients receiving warfarin (Table 3; $p = 0.87$ and $p = 0.98$, respectively).

### *Fill Volume Protocol*

In healthy volunteers, a statistically significant difference among the various fill volumes was demonstrated (Table 4; $p < 0.001$). *Post hoc* tests comparing the INRs from the various tube fills to those from the 100% fill volume revealed a significant difference for the tubes filled only 50% full ($p < 0.01$). Mean INR values from completely full tubes were elevated in three study subjects despite

**Table 3—*Time to Mixing Protocol Results***

| Study Group/Time to Inversions, min | Mean INR (SD) | p Value |
|---|---|---|
| Healthy volunteers (n = 15) | | |
| 0 | 1.1 (0.07) | 0.87 |
| 1 | 1.1 (0.06) | |
| 2 | 1.1 (0.06) | |
| 3 | 1.1 (0.1) | |
| 4 | 1.1 (0.06) | |
| Warfarin patients (n = 15) | | |
| 0 | 3.0 (0.08) | 0.98 |
| 1 | 3.0 (0.8) | |
| 2 | 3.0 (0.8) | |
| 3 | 3.1 (0.8) | |
| 4 | 3.0 (0.8) | |

**Table 4—*Fill Volume Protocol Results***

| Study Group/Fill Volume, % | Mean INR (SD) | p Value |
|---|---|---|
| Healthy volunteers (n = 15) | | |
| 100 | 1.5 (0.9) | < 0.001 |
| 90 | 1.2 (0.2) | |
| 80 | 1.3 (0.2) | |
| 70 | 2.7 (4.2) | |
| 60 | 4.0 (5.6) | |
| 50 | 2.6 (1.1)* | |
| Warfarin patients (n = 15) | | |
| 100 | 3.2 (1.2) | < 0.001 |
| 90 | 3.7 (1.3) | |
| 80 | 4.2 (1.5)* | |
| 70 | 6.4 (3.9)* | |
| 60 | 8.6 (4.3)* | |
| 50 | 9.9 (4.2)* | |

*$p < 0.01$ compared to 100% fill volume.

the fact that the subjects were not receiving warfarin therapy (INR values, 4.2, 2.2, and 3.1, respectively). If these subjects were excluded from the analysis, the INRs from tubes filled < 90% full were significantly higher than those filled 100% full ($p < 0.01$).

In study subjects receiving warfarin therapy, decreasing fill volume also had a marked effect on the measured INR result (Table 4). Analysis revealed that statistically and clinically significant differences in the mean INRs across the six different fill volumes were present ($p < 0.001$). *Post hoc* analysis demonstrated that statistically significant INR differences occurred for evacuated tubes filled < 90% full compared to tubes that were filled to 100% of their volume.

## Discussion

The results of this study demonstrate the potential for falsely elevated INR values derived from blood samples drawn in full-draw pediatric evacuated collection tubes made of siliconized glass that are filled < 90% of full volume. The number of times evacuated tubes were inverted following specimen collection and elapsed time between specimen collection and tube inversion had no effect on INR results provided the tubes were adequately filled. This potential for false INR elevation is especially concerning in light of recent recommendations calling for the routine use of vitamin K to reverse anticoagulation in patients receiving warfarin presenting with symptomless increases in the INR.[4]

Our results are consistent with those of other investigators who also demonstrated prolonged coagulation parameters with decreasing evacuated tube fill volumes.[2] Using 5-mL (adult) evacuated tubes containing 3.2% sodium citrate, it was demonstrated that a minimum of 60% fill volume was required to obtain accurate PT results. When 3.8% sodium citrate was used, a fill volume of ≥ 80% was required to obtain accurate results. It has been hypothesized that "short draws," in which the evacuated tube is underfilled, result in falsely prolonged PT (and hence INR) results because test reagent calcium is neutralized by the relative citrate excess. The amount of citrate in the evacuated tube is fixed. Therefore, when tubes are not completely filled the concentration of citrate (an anticoagulant) is increased resulting in the potential for erroneous INR results. This effect may accentuated by dilution of the plasma by the citrate solution.[5] The explanation underlying why pediatric collection tubes are less forgiving than standard tubes with respect to underfilling is unknown, but we speculate it may relate to a relative increase in plasma dilution due to the citrate solution in the smaller volume tubes.

It is important for anticoagulation providers to be aware that in high-altitude areas, evacuated tubes

**Table 5—*Clinical Impact of Incomplete Collection Tube Volume in Patients Receiving Warfarin***

| Subject No. | 100% Fill INR | 60% Fill INR | Potential Clinical Consequence |
|---|---|---|---|
| 1 | 4.55 | 17.80 | Unnecessary vitamin K administration |
| 2 | 2.50 | 8.85 | Unnecessary omission of warfarin doses or vitamin K administration |
| 3 | 2.20 | 7.25 | Unnecessary omission of warfarin doses or vitamin K administration |
| 4 | 3.25 | 8.10 | Unnecessary omission of warfarin doses or vitamin K administration |
| 5 | 3.65 | 17.80 | Unnecessary vitamin K administration |
| 6 | 3.20 | 5.65 | Unnecessary omission of warfarin doses or vitamin K administration |
| 7 | 3.20 | 5.65 | Unnecessary omission of warfarin doses or vitamin K administration |
| 8 | 6.20 | 7.95 | None |
| 9 | 3.60 | 5.60 | Unnecessary omission of warfarin doses or vitamin K administration |
| 10 | 2.90 | 10.35 | Unnecessary vitamin K administration |
| 11 | 2.50 | 7.65 | Unnecessary omission of warfarin doses or vitamin K administration |
| 12 | 3.95 | 6.40 | Unnecessary omission of warfarin doses or vitamin K administration |
| 13 | 2.45 | 10.45 | Unnecessary vitamin K administration |
| 14 | 2.00 | 4.90 | Unnecessary omission of warfarin doses or reduction in warfarin dose |
| 15 | 1.35 | 2.60 | Failure to increase warfarin dose |

can lose their vacuum due to reduced pressure between the tube and the surrounding atmosphere. Empty tubes stored at high temperatures may also lose vacuum. Both of these conditions can result in underfilled tubes and the potential for inaccurate INR test results.[2]

Relying on inaccurate INR results can lead to clinically important errors in warfarin management decisions as demonstrated in Table 5. Compared to completely full tubes, reliance on the INR results obtained from tubes filled only 60% full would likely have led the anticoagulation provider to implement a different therapeutic plan in 14 of 15 cases. It is reasonable to conclude that the risk for thromboembolism would have been unnecessarily increased in all 14 cases.

Pediatric evacuated tubes are often utilized for patients who have poor venous access as obtaining enough blood to adequately fill an adult tube in these patients can be difficult. Our results emphasize the need to ensure that pediatric evacuated tubes are filled at least 90% full. Pediatric tubes with $< 90\%$ fill volume should be discarded and another sample redrawn. Anticoagulation providers should routinely verify what type of collection tube was used before making therapeutic plans for excessively anticoagulated patients. If a pediatric tube was used and the patient has no symptoms of bleeding, it would be prudent to redraw the sample in an adult evacuated tube if possible. In the absence of bleeding, vitamin K should be withheld until an elevated INR derived from a sample drawn in a pediatric tube is confirmed through repeat testing.

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