

# Serum or Plasma for Quantification of Direct Oral Anticoagulants?

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**Background:** Direct oral anticoagulants are increasingly replacing vitamin K antagonists for prevention of stroke in patients with atrial fibrillation, partly owing to the lack of a need for routine monitoring. Therapeutic drug monitoring may still be warranted under certain circumstances. It is generally assumed that serum and plasma can be interchangeably used for this purpose. The aim of this study was to investigate possible differences between the serum, citrate-plasma, and ethylenediaminetetraacetic acid (EDTA)-plasma concentrations of apixaban and rivaroxaban in a larger patient group and their relation to factor X measurements.

**Methods:** Plasma and serum samples were drawn during the same venipuncture from patients treated with apixaban or rivaroxaban. Drug levels were measured using ultrahigh-performance liquid chromatography combined with tandem mass spectrometry. Three sample matrices were obtained from 8 healthy volunteers for measurement of factor X antigen and activity.

**Results:** Mean concentrations of apixaban and rivaroxaban were 16.8% and 36.6% higher in serum than in citrate-plasma, respectively (both  $P < 0.001$ ). The corresponding differences in serum versus EDTA-plasma were 4.5% for apixaban and 13.1% for rivaroxaban (both  $P < 0.001$ ).

oxaban (both  $P < 0.001$ ). Factor X antigen measurements in citrate-plasma, EDTA-plasma, serum with clot activator, and serum without additives yielded comparable results, and factor X activity was significantly higher in serum than in plasma.

**Conclusions:** Apixaban and rivaroxaban concentrations were significantly higher in serum than in plasma. The difference was more pronounced with rivaroxaban and was larger between serum and citrate-plasma than between serum and EDTA-plasma. Higher factor X activity in serum may explain the observed concentration differences. The choice of matrix is, thus, important when interpreting therapeutic drug monitoring results and in research involving analyses of direct oral anticoagulants. The authors recommend citrate-plasma as the preferred matrix.

**Key Words:** therapeutic drug monitoring, serum, plasma, rivaroxaban, apixaban

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## BACKGROUND

Large phase III clinical trials have demonstrated that direct oral anticoagulants (DOACs) such as dabigatran, rivaroxaban, apixaban, and edoxaban are at least as safe and effective as vitamin K antagonists in the prevention of stroke in patients with nonvalvular atrial fibrillation.<sup>1</sup> Unlike vitamin K antagonists, where the international normalized ratio (INR) can be used to guide dosage, there is no reliable biological measure of effect of DOAC treatment. Thus, therapeutic drug monitoring (TDM) may be a potential option. TDM is based on the assumption that a relationship exists between the serum or plasma concentration of a drug and its clinical effect. This concentration is then related to a therapeutic range, which is associated with the highest possible probability of response combined with a low risk of adverse drug reactions or toxicity.<sup>2</sup> The definition of therapeutic ranges depends on clinical studies linking drug concentrations to clinical outcomes. To date, there are no therapeutic ranges established for DOACs. Instead, reference intervals for DOACs have been proposed based on concentration ranges achieved after intake of the supposedly most effective doses determined in clinical and pharmacokinetic studies.<sup>3–6</sup>

DOACs are marketed without the intention of routine monitoring.<sup>7</sup> The lack of a need for monitoring was based on the assumed interindividual similarities in pharmacokinetic and pharmacodynamic responses combined with a relatively wide therapeutic window.<sup>7</sup> However, clinical trials often

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exclude patients with impaired hepatic or renal functions, children, elderly subjects, those with an increased bleeding risk, those on interacting medications, and those at the extremes of body weight. The lack of a need for monitoring demonstrated in clinical trials may therefore not be applicable to patient groups excluded from trial entry.<sup>7</sup>

Although routine monitoring of DOAC plasma levels is generally not encouraged,<sup>8</sup> TDM is proposed in specific circumstances such as deteriorating renal or hepatic function and in the elderly and frail.<sup>8</sup> Other suggested indications for TDM are treatment failure, suspected overdose, bleeding, possible drug interactions, and in patients presenting with acute ischemic stroke while on a DOAC, particularly in those for whom thrombolysis is being considered.<sup>7,8</sup> In addition, TDM of DOACs could be helpful in the assessment of patient adherence or in patients with intestinal malabsorption, for instance after bariatric surgery.

Studies undertaken to investigate DOAC pharmacokinetics have used plasma as a matrix,<sup>3–5</sup> as opposed to serum. It is generally assumed that serum and plasma can be interchangeably applied for TDM.<sup>9</sup> However, considering the drugs' mechanism of action of binding to coagulation factors, this may not necessarily be the case for DOACs. In fact, Harenberg et al<sup>10,11</sup> found about 20%–25% higher apixaban and rivaroxaban concentrations in serum than in citrate-plasma. However, drug concentrations in these studies were not directly measured using liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) methods but with chromogenic methods. Chromogenic methods are based on adding an excess of activated factor X (FXa) to the patient plasma and then measuring the amount of FXa not bound to DOAC, that is, an indirect measurement of DOAC concentration.<sup>10,11</sup> We are not aware of any other studies investigating this topic, particularly with the use of ultrahigh-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) measurement of DOACs combined with enzyme-linked immunosorbent assay (ELISA) measurement of factor X (FX) antigen and a clot-based method of factor X activity in different sample matrices.

The aim of the present study was to investigate possible differences between serum, citrate-plasma, and ethylenediaminetetraacetic acid (EDTA)-plasma concentrations of apixaban and rivaroxaban in a larger patient group, and the potential influence of calibrator matrices (serum or citrate-plasma) through direct analysis of drug concentrations by UHPLC-MS/MS. Differences in concentrations were then related to the measurement of FX antigen concentrations and activities in different sample matrices.

## MATERIALS AND METHODS

### Samples

Plasma and serum samples drawn during the same venipuncture from patients treated with apixaban or rivaroxaban were obtained from 3 different sources as follows:

1. EDTA-plasma and serum parallel samples (70 apixaban, 20 rivaroxaban) were collected from patients included in the “Norwegian cognitive impairment after stroke study” (Nor-COAST).<sup>12</sup> As the samples were collected from

several hospitals, specific types of EDTA-plasma and serum tubes used were unknown. Centrifugation was performed according to local procedures at each hospital. Aliquots of serum and plasma samples were frozen in separate tubes at inclusion hospitals, transported on dry ice, and stored at  $-80^{\circ}\text{C}$  at BioBank1, Central Norway Health Authority.

2. Citrate-plasma and serum parallel samples (30 apixaban, 8 rivaroxaban) were collected from patients included in the study “Safer treatment with direct oral anticoagulants in nursing home patients,” a pharmacological and biochemical monitoring study (GeriDrug) involving several nursing homes in Bergen, Norway. Samples were collected into 6-mL BD Vacutainer serum tubes with clot activator (CAT; Clot Activator Tube) and 3-mL BD Vacutainer citrate tubes with 9NC 0.109 M buffered trisodium citrate. After 30–60 minutes, serum tubes were centrifuged for 12 minutes at 1500g, and serum was transferred into separate tubes. Citrate-plasma tubes were immediately centrifuged for 15 minutes at 2500g, and the plasma was transferred into separate tubes. The tubes were then transported in a refrigerated state to Haukeland University Hospital and kept overnight at  $-20^{\circ}\text{C}$  before being stored at  $-80^{\circ}\text{C}$  in Biobank Haukeland.
3. Citrate-plasma and serum parallel samples (17 apixaban and 7 rivaroxaban) were collected from patients admitted to St. Olav University Hospital with symptoms of stroke or transient ischemic attack. These patients were included in a study examining serum concentrations of DOACs in this patient group. Samples were collected in 3.5-mL Greiner Vacuette citrate tubes with 3.8% sodium citrate and in 6-mL Greiner Vacuette tubes with CAT serum clot activator. Citrate-plasma tubes were immediately centrifuged for 15 minutes at 2500g, and the plasma was transferred into separate tubes and stored at  $-80^{\circ}\text{C}$ . Serum tubes were centrifuged after 30 minutes for 10 minutes at 2200g, and the serum was transferred into separate tubes and stored at  $-80^{\circ}\text{C}$ .

Informed consent was obtained from all patients before inclusion in the study. All original studies were approved by the Regional Ethics Committee (REK) with the registration numbers 2017/1462 REK Nord (source 1), 2018/1414 REK Vest (source 2), and 2018/297 REK Midt (source 3).

### Analysis of Apixaban and Rivaroxaban

Plasma and serum concentrations of apixaban and rivaroxaban were analyzed using the UHPLC-MS/MS method. The analytical method used for serum as the matrix has been previously published<sup>13</sup> and was revalidated for plasma for the present study. In brief, sample preparation was performed using a Hamilton Microlab STAR automated liquid handler (Hamilton Robotic, Bonaduz, Switzerland). The sample preparation (100  $\mu\text{L}$ ) included a protein precipitation (375  $\mu\text{L}$  acetonitrile with 1% formic acid) and filtration step using an Ostro 96-well plate (Waters, Milford, MA). The eluates were collected in 96-well plates. Separation was performed on an Acquity UPLC I-Class FTN system (Waters, Milford, MA) at  $50^{\circ}\text{C}$  with a UPLC HSS T3 (1.8 mm,  $2.1 \times 100$  mm) analytical column (Waters). The mobile phase comprised MilliQ  $\text{H}_2\text{O}$  with 0.1% formic acid (A)

and acetonitrile (B) at a flow of 0.6 mL/min. Analytes were eluted with a linear gradient from 95% A to 98% B in 3.0 minutes. The injection volume was set at 1  $\mu$ L. MS detection was performed on a Xevo TQ-S tandem quadrupole MS (Waters, Manchester, United Kingdom) using positive electrospray ionization. The monitored mass-to-charge ratio transitions for apixaban and rivaroxaban were  $m/z$  460.3 > 199.0 and  $m/z$  436.1 > 145.0, respectively, whereas the multiple reaction monitoring transitions for apixaban- $^{13}\text{C}_7$  and rivaroxaban- $^{13}\text{C}_6$  were  $m/z$  468.2 > 199.1 and  $m/z$  442.1 > 145.0, respectively.

Human blank serum, citrate-plasma, and EDTA-plasma were obtained from healthy blood donors without anticoagulant use (St. Olav University Hospital, Trondheim, Norway). The validation methodology was previously published.<sup>13</sup> The calibrated range for both apixaban and rivaroxaban in the serum and plasma was 5.0–800 nmol/L, equal to 2.3–368 ng/mL for apixaban and 2.2–349 ng/mL for rivaroxaban. Calibrators and controls were prepared using blank serum and citrate-plasma. In addition, calibrators and controls with the same calibration range were prepared in blank EDTA-plasma. The purpose was to compare concentrations in EDTA-plasma and citrate-plasma from patients upon analysis with a standard curve spiked in another matrix than the sample matrix.

The limit of quantification (LOQ) was determined at the lowest calibrator level (5.0 nmol/L). Linearity was observed throughout the calibrated intervals ( $R > 0.985$ ). Table 1 presents the validation data for the serum and citrate-plasma.

### Factor X Antigen and Activity in Healthy Volunteers

Citrated blood, EDTA blood, and serum samples were obtained from 8 healthy volunteers in the research group. This study was approved by the Regional Ethics Committee (registration number 2018/1414/REK Vest). Citrated-blood [Vacuette 0.109 mmol/L (3.2%) (Greiner Bio-One GmbH, Krefeld, Austria)] was centrifuged at 2500g for 15 minutes and EDTA-blood [Vacuette K2EDTA (Greiner Bio-One)] and serum [Vacuette serum with clot activator (Greiner Bio-One)] at 2200g for 10 minutes. The serum tubes were kept idle for 30 minutes before centrifugation. In 3 of the volunteers, an additional sample was drawn in a plain

serum glass tube [BD Vacutainer serum without additives (Becton Dickinson and company, NJ)], and these tubes were kept idle for 1 hour before centrifugation at 1400g for 12 minutes. Samples were aliquoted and frozen within 1 hour (1.5 hours for the serum tube without additives).

FX antigen was measured using ELISA (Zumutest Factor X; Hyphen Biomed, Neuville-sur-Oise, France) on a Versamax microplate reader (Molecular Devices, San Jose, CA). FX activity was measured with a clot method in seconds (Prothrombin time Quick reagent RecombiPlasTin; Instrumentation Laboratory, Bedford, MA) on ACL TOP 700 (Instrumentation Laboratory), calibrated to give results in IU/dL.

### Statistical Analyses

When comparing concentrations in the plasma and serum, relative differences are reported as the mean of the individual relative differences, and the 95% confidence interval of the mean, with plasma as the reference. As drug concentrations were not normally distributed, the Wilcoxon signed-rank test was used to assess the statistical significance of concentration differences between matrices. Statistical significance was set at  $P < 0.05$ . Statistical analyses were performed using SPSS version 27 (IBM, Armonk, NY).

## RESULTS

The median measured concentrations of apixaban and rivaroxaban and the relative concentration differences in serum versus citrate-plasma and serum versus EDTA-plasma are presented in Table 2. Both apixaban and rivaroxaban concentrations were significantly higher in serum than in EDTA-plasma and citrate-plasma ( $P < 0.001$  for all comparisons). The concentration difference was most pronounced for rivaroxaban and larger between the serum and citrate-plasma than between the serum and EDTA-plasma.

The individual, relative concentration differences between the serum and plasma (citrate or EDTA) are shown in Figure 1. For apixaban, the relative concentration difference between the serum and EDTA-plasma tended to be larger with higher absolute concentrations. No such trend

**TABLE 1.** Extraction Recovery, Matrix Effects, and Interassay and Intraassay Precision Data for Apixaban and Rivaroxaban in the Serum and Citrate-Plasma

Analyte	Quality Control Concentration (nmol/L)	Recovery (%)		Matrix Effects* (%)		Interassay Precision (%CV, n = 6)		Intraassay Precision (%CV, n = 6)	
		Serum†	Plasma	Serum†	Plasma	Serum†	Plasma	Serum†	Plasma
Apixaban	13.0	102	103	88	96	4.8	5.0	4.6	3.6
	75.0	—	—	—	—	4.6	7.1	10.0	4.4
	640	98	100	102	104	4.4	7.4	5.1	4.2
Rivaroxaban	13.0	105	109	95	104	3.7	3.1	7.1	3.2
	75.0	—	—	—	—	4.9	9.2	9.0	3.5
	640	100	104	98	94	3.6	5.7	3.3	2.6

\*Internal standard-normalized values.

†Serum values have been previously published.<sup>12</sup>

**TABLE 2.** Median (Range) Concentrations of Apixaban and Rivaroxaban in the Serum and Plasma, and Relative Concentration Differences Between the Serum and Plasma (Citrate-Plasma, Upper Panel; EDTA-Plasma, Lower Panel)

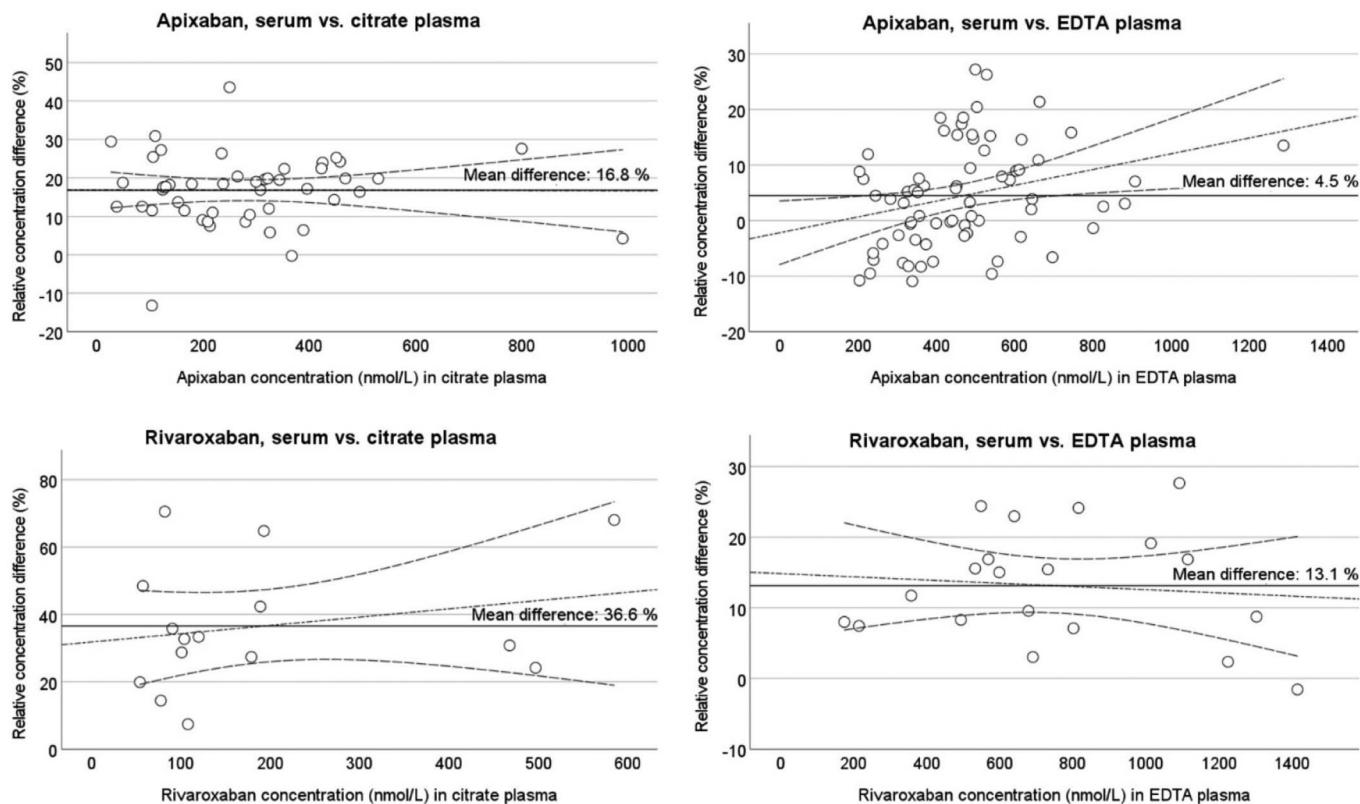
Drug	N	Serum Concentration (nmol/L), Median (Range)	Plasma Concentration (nmol/L), Median (Range)	Relative Difference, Serum Versus Plasma	
				Mean (Range)	95% Confidence Interval
Serum versus citrate-plasma					
Apixaban	47	318 (35–1032)	265 (27–990)	16.8% (–13.5%–43.6%)	14.1%–19.5%
Rivaroxaban	15	140 (65–983)	108 (54–585)	36.6% (7.4%–70.7%)	26.0%–47.1%
Serum versus EDTA-plasma					
Apixaban	70	484 (182–1461)	460 (204–1287)	4.5% (–10.9%–27.2%)	2.3%–6.7%
Rivaroxaban	20	766 (189–1417)	685 (175–1415)	13.1% (–1.6%–27.7%)	9.4%–16.9%

All differences were statistically significant ( $P < 0.001$ ).

was observed for apixaban between the serum and citrate-plasma or for rivaroxaban.

Results of comparisons of concentrations in patient samples after analysis with a standard curve spiked in another matrix than in the sample matrix are presented in Table 3. Overall, the differences in the concentrations when calibrated with different matrices were negligible.

In samples from 8 DOAC-free healthy volunteers, the median (range) FX antigen concentrations in citrate-plasma, EDTA-plasma, serum with clot activator, and serum without additives were 67.9 (59.6–84.3), 82.4 (69.5–103.0), 78.4 (69.7–95.3), and 69.6 (68.0–75.4) IU/dL, respectively (Fig. 2A). The median FX antigen concentration in the citrate-plasma was 11.5% (range, 8.5%–15.9%) lower than that



**FIGURE 1.** The relative concentration differences between serum and citrate-plasma (left) and serum and EDTA-plasma (right) for apixaban and rivaroxaban, plotted against the absolute concentration in plasma. The solid line represents the mean relative difference between the matrices. The broken lines represent the linear correlation (straight line) with 95% confidence interval (curved lines).

**TABLE 3.** Mean (Range) Relative Concentration Difference When Patient Samples Were Analyzed With a Standard Curve Spiked in a Matrix Other Than the Sample Matrix

Patient Samples	Standard Curve		
	Citrate Plasma	EDTA Plasma	Serum
Apixaban	Citrate-plasma	Reference	+1.8% (+1.1 to +2.1%)
	EDTA-plasma	−0.3% (−1.2 to +1.7%)	Reference
Rivaroxaban	Citrate-plasma	Reference	+1.3% (−0.6 to +2.3%)
	EDTA-plasma	NA	Reference

Positive numbers represent concentrations higher than the concentration obtained with the reference plasma, and negative numbers represent concentrations lower than the concentration observed with the reference plasma. The numbers of patient samples tested were 5 for the citrate-plasma and 10 for the EDTA-plasma for apixaban and 5 for the citrate-plasma for rivaroxaban.  
NA, not applicable.

in the serum with clot activator. The median (range) FX activities in the citrate-plasma, EDTA-plasma, serum with clot activator, and serum without additives were 77.6 (70.4–93.4), 91.2 (83.0–105.9), 263.3 (184.0–313.1), and 158.4 (153.3–158.4) IU/dL, respectively (Fig. 2B). The median (range) FX activity in the citrate-plasma was 70.2% (59.1%–73.3%) lower than that in the serum with clot activator.

## DISCUSSION

The key finding of the present study is the significantly higher apixaban and rivaroxaban concentrations in serum than in both EDTA-plasma and citrate-plasma. The concentration difference was most pronounced for rivaroxaban and larger between the serum and citrate-plasma than between serum and EDTA-plasma. Only 10% of the difference between serum and citrate-plasma can be attributed to the dilution effect in the citrate-plasma.

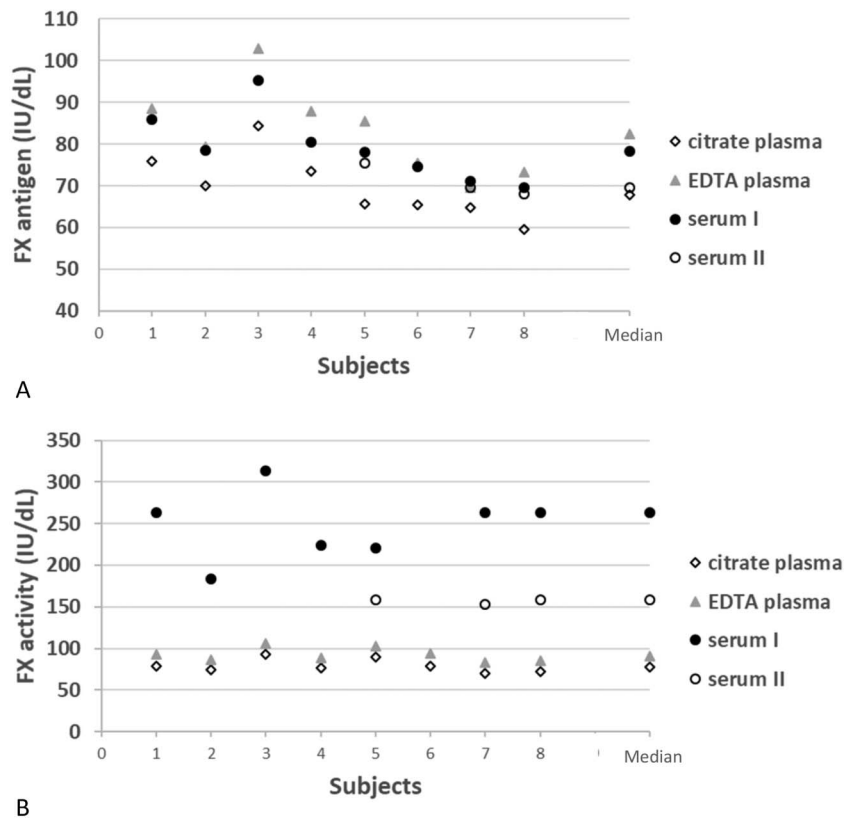
These results expand our understanding of matrix differences as compared with those reported in previous studies,<sup>10,11</sup> in which both apixaban and rivaroxaban concentrations were approximately 20%–25% higher in serum than in citrate-plasma. In our study, the corresponding difference was 15%–20% for apixaban and 35%–40% for rivaroxaban. In previous studies,<sup>10,11</sup> concentrations of apixaban and rivaroxaban were indirectly measured by chromogenic assays, as opposed to a direct analytical method (UHPLC-MS/MS) used in the present study. Whether the discrepancies in concentration differences for rivaroxaban between previous studies and the present report are caused by differences in the analytical methods applied or are just coincidence remains unanswered.

In DOAC-free healthy volunteers, FX seemed to remain in both serum and plasma after centrifugation (ie, not being trapped among the eliminated cells) because FX antigen was almost similar in different matrices (when corrected for 10% dilution in citrate-plasma) (Fig. 2A). However, FX activity in serum with clot activator was more than twice the activity reported in both citrate-plasma and EDTA-plasma (Fig. 2B). This observation is probably explained by the coagulation activation starting as soon as the blood comes in contact with the clot activator or the glass wall in the serum tube, thereby activating coagulation factors, including conversion of FX into FXa. In plasma, however, inhibitors of

coagulation (EDTA and citrate, which bind the calcium needed to induce coagulation) ensure inhibition of coagulation activation and FX is, therefore, maintained in its inactivated form. As DOACs directly bind to FXa, it is reasonable to believe that the higher measured DOAC concentrations in the serum are due to the increased binding of DOACs to FXa because FX activity is higher in the serum than in the plasma. Notably, both in TDM and in regular research analyses, total concentrations of free + protein-bound drugs (including plasma proteins such as albumin, orosomucoid, coagulation factors, and drug-specific transport proteins) are measured. Furthermore, one may speculate that the “extra” DOAC found in the serum is owing to its diffusion from the cells *in vitro* in case of increased FX activity in serum. In the plasma, however, the lower FX activity in the tube reduces the diffusion of DOACs from the cells. This theory, even after considering the dilution effect in the citrate-plasma, cannot fully explain the lesser concentration difference between serum and EDTA-plasma than between serum and citrate-plasma because FX activity is almost similar in citrate-plasma and EDTA-plasma. We cannot exclude the possibility that other types of tubes such as serum tubes lacking clot activator were used for sample collection in the first study. If so, this might contribute to explaining the lesser difference in FXa between the serum and EDTA-plasma.

From our results, it is obvious that the choice of matrix must be considered when evaluating TDM results and when interpreting data on DOAC concentrations from studies. In TDM practice, it is important that the sample matrix type is registered before analysis so that the clinician is aware that the choice of the matrix can in itself be a source of error. In previously published clinical studies, both analytical methods used and the choice of matrix varied. In some studies, DOAC concentrations were indirectly measured by chromogenic methods.<sup>14,15</sup> In studies where DOAC concentrations were directly quantified, different types of matrices were used. Some studies have used citrate-plasma<sup>3,16</sup> or Li-heparin plasma,<sup>17</sup> whereas others have not clearly specified the type of plasma applied.<sup>5,18</sup>

Traditionally, TDM in Norway and many other countries is conducted using serum. The results of this study, together with the fact that large clinical phase III studies have used plasma<sup>19–21</sup> and that citrate-plasma is the matrix generally



**FIGURE 2.** Factor X (FX) antigen concentrations (A) and FX activity (B) measured in citrate-plasma, EDTA-plasma, and serum [with (serum I) and without (serum II) clot activator] from 8 healthy volunteers.

used for coagulation tests, have led us to conclude that citrate-plasma should be preferred for the analysis of DOACs.

Although studies have shown that samples for DOAC monitoring with functional coagulation assays can be stored for 24 hours at room temperature before testing without loss of stability,<sup>22</sup> in vitro stability of DOACs stored at room temperature is significantly longer.<sup>13</sup> Direct measurement of DOAC concentration in plasma samples by chromatographic methods may, therefore, be necessary for monitoring treatment with DOACs outside the hospital setting when such quantification is indicated.

This study has some limitations and some strengths that should be acknowledged. The number of patients recruited was relatively small, particularly in the rivaroxaban group. Nevertheless, all results were statistically highly significant, with correspondingly narrow confidence intervals. Although some individuals had lower apixaban levels in serum than in EDTA-plasma (Fig. 1, upper right panel), the analytical variation of the method may explain this finding. This finding does not, however, contradict the fact that the concentration difference between serum and EDTA-plasma was statistically significant, albeit small. One may also speculate that the degree of anticoagulation by citrate and EDTA may be affected by interindividual variations in (the concentrations of) endogenous substances.

Another limitation of this study is that we, for practical reasons, were unable to obtain citrate-plasma and EDTA-plasma from the same blood sampling. Thus, no direct comparison could be made between plasma samples with

these 2 additives. Standard curves in different matrices showed very small changes in measured drug concentrations (Table 2). As these changes were almost negligible as compared with the mean relative differences in drug concentrations between the serum and plasma, we feel confident that the choice of matrix for standard curves will have no significant effect on measured concentrations. It should also be noted that we did not test the recoveries, matrix effects, and analytical precision in EDTA-plasma.

The strengths of this study are the comparison of drug concentrations in parallel serum and plasma samples from patients rather than the spiked material and the comparison of matrices for calibrator preparation. Moreover, we suggest a possible explanation for the differences in DOAC concentrations dependent on the additive by demonstrating differences in FX antigen concentration and activity in the various matrices tested.

### CONCLUSIONS

Apixaban and rivaroxaban concentrations were significantly higher in serum than in EDTA-plasma and citrate-plasma. The concentration difference was most pronounced with rivaroxaban, and larger between serum and citrate-plasma than between serum and EDTA-plasma. We suggest that the observed differences may be caused by increased binding of DOACs to FXa, as FX activity was higher in serum than in plasma. We recommend citrate-plasma as the preferred matrix for the analysis of DOAC concentrations.

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