ONLINE SUPPLEMENTARY CONTENT

Validation of the use of idarucizumab in the internal calibrator of dabigatran samples for the thrombin generation assay

Control experiments, using a thrombin time test, showed that 1 mg/mL idarucizumab neutralised up to 2 µg/mL dabigatran (the highest tested concentration, not shown).

To validate our thrombin generation (TG) assay, we tested normal plasma spiked with 150 ng/mL dabigatran, which allowed us to use the very same plasma without dabigatran as an internal calibrator (to avoid the inhibition of thrombin-$\alpha_2$macroglubulin complex). The assay was carried out exactly as for patients' plasma, using 6 pM tissue factor (TF).

In the first set of experiments, we evaluated TG in dabigatran-spiked plasma using three different calibrators: (i) normal plasma (NP); (ii) NP spiked with dabigatran (150 ng/mL) and (iii) NP spiked with dabigatran plus 1 mg/mL idarucizumab. As shown in **Figure S1A**, the TG curves calculated using as internal calibrators NP or NP containing dabigatran plus idarucizumab were perfectly superimposable, whereas the curve recorded with dabigatran-containing plasma as calibrator was strikingly different, showing a much higher thrombin peak and greater endogenous thrombin portential. These findings indicate that the addition of idarucizumab to the internal calibrators efficiently prevents the artefact related to the inhibition of thrombin-$\alpha_2$macroglubulin by dabigatran. Moreover, they show that the excess of idarucizumab used in our assays does not interfere with the cleavage of the fluorogenic substrate by thrombin-$\alpha_2$macroglubulin complex.

Next, to further investigate the reliability of our method for TG assay in dabigatran samples, we compared the TG curves obtained with the fluorometric method and with a two-stage assay insensitive to thrombin-$\alpha_2$macroglubulin complex, which is based on the measurement of thrombin activity by a clotting test[s1]. Briefly, plasma was challenged with 6 pM tissue factor, after which subsamples were taken at predetermined intervals for measurement of thrombin activity using purified fibrinogen as substrate. Clotting times were then converted to thrombin activity (U/mL) by reference to a calibration curve constructed with purified human thrombin (Haematologic Technologies, Essex Junction, VT, USA). Experimental conditions were identical to those used for the fluorometric assay, except that plasma was defibrinated to facilitate subsampling.

A representative experiment with plasma spiked with 150 ng/mL dabigatran is illustrated in **Figure S1B**. The TG curve recorded with the two-stage clotting method was very similar to that calculated by the fluorometric assay, even though it was slightly shifted to the left. Assuming a thrombin-specific activity of 3000 U/mg, we calculated a thrombin peak of approximatively 190 nM, which is somewhat lower than that calculated by the fluorometric assay (250 nM). These slight differences might be due, at least partly, to the use of defibrinated plasma in the two-stage assay; moreover, considering that the minimum interval between two consecutive determinations of thrombin activity was 2 min (as opposed to 20 s for the fluorometric assay), it is possible that we missed the true thrombin peak.

These findings suggest that the fluorometric TG assay of samples from patients under dabigatran treatment provides more reliable results when idarucizumab is added to the sample-specific calibrator. Furthermore, even assuming that the absolute values of the TG parameters could be slightly different from the true ones, it can be safely concluded that the behaviour of dabigatran samples is clearly different from samples containing the anti-Xa direct oral anticoagulants (DOAC) (see below).

Effect of tissue factor concentration on thrombin generation in plasma from patients under treatment sith direct oral anticoagulants

Four patients' samples from each DOAC group, taken at peak drug level, were tested using 6 pM and 20 pM TF. TG was evaluated according to Hemker *et al.*[s2], with some modifications as detailed in the Materials and Methods of the manuscript. As shown in **Figure S2**, on addition of 20 pM TF (panel B), besides the obvious changes in TG parameters (especially thrombin peak), the different behaviour of dabigatran compared to anti-Xa direct oral anticoagulants was as evident as in the presence of 6 pM TF (panel A). Indeed, under both conditions, dabigatran plasma displayed a rapid increase of thrombin activity, a high peak level and a rapid decay of the enzyme, whereas the anti-Xa samples displayed more flattened TG curves.

SUPPLEMETARY REFERENCES

s1. Incampo F, Carrieri C, Semeraro N, Colucci M. The paradoxical antifibrinolytic effect of dabigatran and argatroban in the presence of soluble thrombomodulin is unrelated to protein C-dependent increase of thrombin generation. Thromb Res 2014; **134**: 1110-6.

s2. Hemker HC, Al Dieri R, De Smedt E, Béguin S. Thrombin generation, a function test of the haemostatic-thrombotic system. Thromb Haemost 2006; **96**: 553-61.

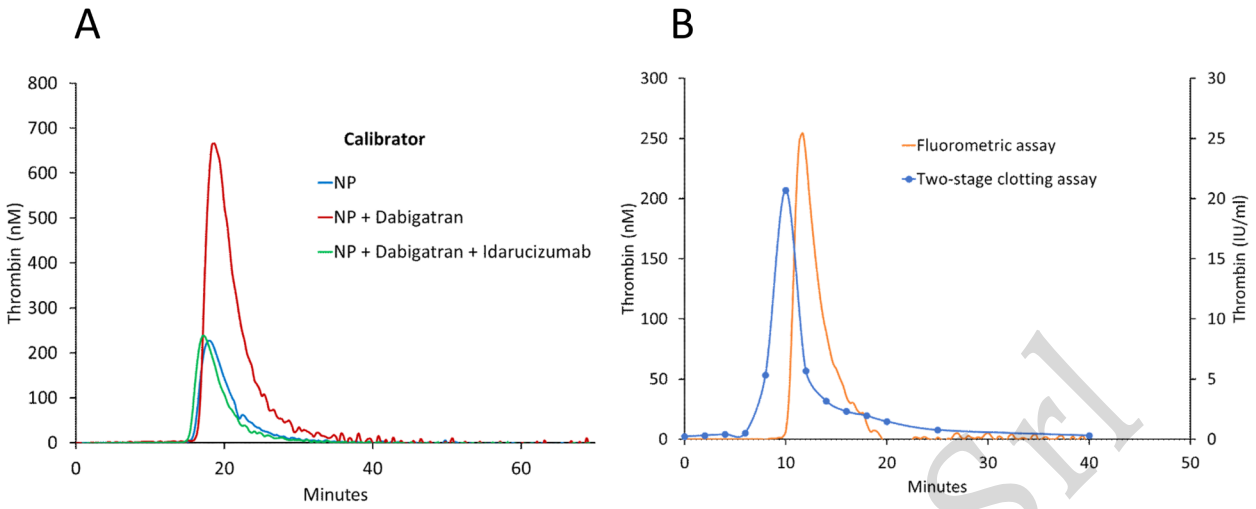

Figure S1 - Validation of the use of idarucizumab in the internal calibrator of dabigatran samples for the thrombin generation (TG) assay
(A) TG curves in plasma spiked with 150 ng/mL dabigatran using different internal calibrators. (B) TG curves recorded using the fluorometric assay and the two-stage clotting assay. Experiments were performed using 6 pM tissue factor as the clotting trigger. Each graph shows TG curves representative of three independent experiments with similar results. NP: normal plasma.

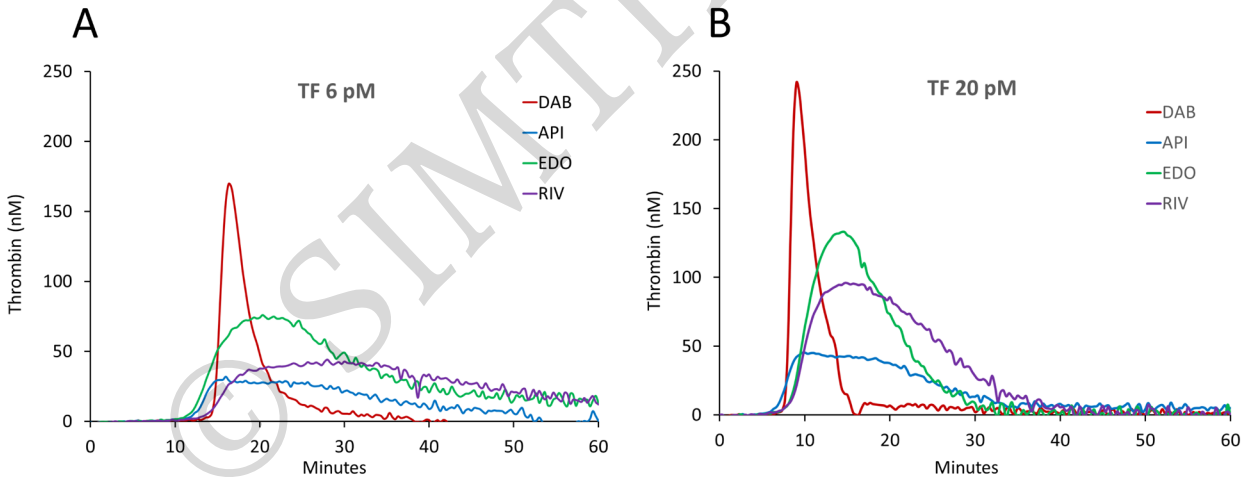

Figure S2 - Thrombin generation in plasma from patients under treatment with direct oral anticoagulants tested with two different tissue factor concentrations, 6 pM (panel A) and 20 pM (panel B)
Each graph shows thrombin generation curves representative of four independent experiments in different patients.

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