Pre-analytical biases in investigating low-dose aspirin responsiveness and comparison of two different methods

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Introduction. Low-dose aspirin (ASA) irreversibly inhibits cyclooxygenase (COX)-1 in circulating platelets by blocking thromboxane (TX) A_2synthesis mainly in the portal blood (Patrono. N Engl J Med. 1994). TXA_2 induces platelet aggregation and vasoconstriction an is a short-lived prostanoid, non-enzymatically converted to TXB_2. Measurement of serum TXB_2 is an*ex vivo*index of the maximal biosynthetic capacity of platelet's COX-1 (Patrono et al. Thromb Res.1980),indicated by the European Medicines Agency as the biomarker, surrogate of efficacy, needed for the approval of the new formulations of low-dose ASA (www.ema.europa.eu). The serum TXB_2 assay relies on the physiological generation of endogenous thrombin during*in vitro*blood clotting, which maximally activates platelet COX-1 and subsequent TXA_2 synthesis (Patrono et al. Thromb Res.1980). In the original method, 37°C incubation is necessary. However, the need for immediately incubating blood samples at 37°C might limit the feasibility of this assay in large studies. In fact possible delays between blood withdrawal and blood incubation at 37°C might derive from distance between the outpatient basis and the laboratory and/or multiple blood tubes from the same or different patients kept over the counter after withdrawal before their final destination.

Objectives. 1) To validate the immunologic measurement (EIA) of serum TXB_2 performed in our laboratory according to the original method, with liquid chromatography-tandem mass spectrometry (LC-MS/MS). 2) To investigate if a delay in starting 37°C incubation might affect serum TXB_2 values.

Methods. EIA was performed as previously described (Pascale et al. Blood. 2012) with a previously characterized anti-TXB_2 antibody (Pradelles et al. Anal Chem. 1985). LC-MS/MS was performed according to standard method. To investigate the effects of the delay in the incubation, peripheral blood from 43 volunteers was withdrawn into glass tubes without anticoagulant. One tube was immediately placed at 37°C for 1 hr (reference sample). The remaining tubes were stored at room temperature (RT) from 5 up to 30 min and then incubated at 37°C for 1 hr. Some samples were incubated at RT for 1 hr. Clotted blood samples were centrifuged at 1,200 g for 10 min and supernatant serum was stored at -20°C. $TXB_2$was measured by previously- described EIA (Pascale et aql. Blood. 2012).

Results. There was a high correlation between TXB_2values measured by EIA and LC-MS/MS methods: rho=0.947, $p<0.0001$ (n=46). Serum TXB_2 by EIA method averaged: 2.3[1.25-3.77]ng/ml; serum TXB_2 by LC-MS/MS averaged: 2.4[2.10-3.47]ng/ml. In the studies of delayed incubation at 37°C, data were analyzed as % decrease of the correspondent reference sample. The analysis of data expressed as % of serum TXB_2in the reference samples showed an exponential decrease of serum TXB_2values as a function of time: 5 min= 6% decrease, 15 min=35%, 30 min= 50%, 1 hr=77%. Correlations between serum TXB_2values in reference samples and in samples incubated at each time point were significant (all $p<0.05$), however the slopes of the linear fitting between absolute TXB_2 values measured in the reference samples vs the correspondent samples with delayed incubation progressively decreased as a function of time (5 min: slope=0.95; 10 min: slope=0.92; 15 min: slope=0.83; 20 min: slope=0.58; 30 min: slope=0.56; 1 hr: slope=0.08).

Conclusions. The measurement of serum TXB_2 with EIA method is consistent with LC-MS/MS measurements. A delay ≥ 5 min in starting 37°C incubation of blood samples might underestimate serum TXB_2values. An incorrect pre-analytical handling might thus generate relevant pre-analytical biases which might be critical when assessing the responsiveness to low-dose ASA.