An optimised, rapid chromogenic assay, specific for measuring direct factor Xa inhibitors (rivaroxaban) in plasma

Meyer Michel Samama1,2; Jean Amiral3; Céline Guinet2; Elisabeth Perzborn4; François Depasse2

1Hôtel-Dieu University Hospital, Paris, France; 2Biomnis Laboratories R&D, Ivry sur Seine, France; 3HyphenBiomed, Neuville sur Oise, France; 4Bayer HealthCare AG, Wuppertal, Germany

Dear Sirs,

To improve existing strategies for the prevention and treatment of thromboembolic diseases, new drugs targeting specifically clotting serine proteases (especially factor Xa [FXa] or thrombin) have been introduced recently (1–3). In the future, it is anticipated that long-term anticoagulant therapy will favour oral agents with a wide therapeutic window and a predictable anticoagulant response that do not require routine coagulation monitoring or dose adjustment. However, a quantitative determination of plasma concentrations might be valuable in some cases, such as severe overdose or to assess compliance. Therefore, specific laboratory assays, easily applied to automatic laboratory instruments to measure these new drugs in plasma, are required.

Conversely to heparins and fondaparinux, which express an indirect anti-FXa catalytic activity in presence of antithrombin (AT) (4), direct FXa inhibitors (DFXals) bind directly to the active site.

Various kinetics methods (endogenous antithrombin) and two stage assays (exogenous antithrombin) are used for LMWH and fondaparinux (4, 5). They could be adapted to measure oral DFXals such as rivaroxaban and are based on the inhibition of a constant and in excess amount of FXa in presence of AT (4, 5). However, without modification they are appropriate only for the lower concentration range of rivaroxaban, and are not specific for this drug class. Furthermore, all current anti-FXa assays proposed for testing LMWH provide results expressed in heparin anti-FXa units, which is not appropriate for DFXals. Inhibition kinetics of indirect (catalytic) FXa inhibitors is very different compared to direct reversible inhibitors. Especially, with the two stage assays, the first incubation step has an incidence on the FXa inhibition level, as the result of the catalytic mode of action of indirect inhibitors, which act through AT. Therefore, an assay measuring over a broader concentration range and specific calibrators and control plasmas are required.

A rapid assay, specific for DFXals, without interference of plasma factors concentration and of indirect polysaccharide type inhibitors was developed. The assay is based on inhibition of a constant and in excess concentration of human FXa (about 5

Figure 1: Concentration response curves with rivaroxaban (A), fondaparinux (B) or enoxaparin (B) on the anti-FXa activity using the new assay.
µg/ml), which offers high sensitivity and accuracy at plasma concentration from 0.02 to 0.50 µg/ml (1.145 µM) of rivaroxaban with a linear dose-response curve and no interference of plasma proteins, or indirect FXa inhibitors (Fig. 1). The assay is performed in a high ionic strength pH 7.90 buffer, inhibiting the catalytic action of heparins/fondaparinux, but without effect on the FXa inhibition by rivaroxaban. Diluted plasma (1:20) is incubated with human FXa (Hyphen BioMed, Neuville sur Oise, France) for 60 to 120 seconds at 37°C, then a specific FXa substrate [CS-11(32)] (Hyphen BioMed) is added and the colour development is measured. Calibration is performed using a reference preparation of rivaroxaban (from 0 to 0.50 µg/ml) spiked in plasma. With the microtiter plate (MTP) method, the absorbance at 405 nm decreased from 1.40 to 0.40 (r² = 0.998). The new method was evaluated according to CLSI guidelines (ex-NCCLS), and statistics were performed using the “Analyse-it” software.

Similar curves were obtained when drug was spiked in the dilution buffer or in plasma. The limit of quantitation in plasma was calculated at 0.02 µg/ml. The assay is highly flexible, and a lower limit of detection of 0.01 µg/ml is achieved using lower plasma dilutions (1:10)

The coefficient of variation (CV) in 20 plasma samples spiked with 0.10 and 0.30 µg/ml rivaroxaban were 6.9% and 4.2% for the within-run and 7.2% and 4.1% for the between-run, respectively. Recovery of rivaroxaban spiked into plasma was close to 100%, whether tested in normal, pathological prothrombin or factor X (FX) -deficient plasma. This assay was used in a multicentric quality assessment study (RiVaMoS, involving eight different centres), for measurements of rivaroxaban in plasma, and concentrations obtained were comparable to those measured by HPLC (6). However, additional studies in patients’ plasma with the new assay and those determined with HPLC method are necessary to confirm the assay specificity.

A modified prothrombin time could be used for the measurement of FXa direct inhibitors, although it is not specific (7). The new method is specific and highly sensitive. It uses diluted plasma (which makes the assay very flexible for any dynamic range to be assayed), has no interference from plasma proteins, and is specific without interference from heparin-like substances. This might be helpful in cases of switching therapy from heparin to rivaroxaban. In addition the assay is automatable on laboratory instruments, and can also be used on MTPs in any clinical laboratory equipped with a coagulation instrument or a photometric instrument for enzymatic assays.

In eight healthy white male subjects, and in 135 patients from phase IIb studies, receiving 10 mg tablets of rivaroxaban for thromboprophylaxis in orthopaedic surgery, the maximal plasma concentrations ranged from 0.112 to 0.184 µg/ml and from 0.091 to 0.195 µg/ml, respectively (8, 9). This range of concentrations and even higher concentrations up to 0.5 µg/ml are well covered with this method.

References