Paramagnetic micro-particles as a tool for rapid quantification of apixaban, dabigatran, edoxaban and rivaroxaban in human plasma by UHPLC-MS/MS

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Abstract

Background: Assessment of the anticoagulant activity of direct oral anticoagulants (DOACs) is justified in special clinical situations. Here, we evaluated two independent extraction methods and developed a multi-analyte ultra-high performance liquid chromatography tandem mass (UHPLC-MS/MS) method for the quantification of apixaban, dabigatran, edoxaban and rivaroxaban in human plasma.

Methods: Routine extraction based on protein precipitation with acetonitrile and subsequent centrifugation was compared to sample clean-up using commercial paramagnetic micro-particles and subsequent magnetic depletion. Stable isotope-labeled analogs of all analytes were employed as internal standards. The method was validated according to international guidelines in terms of linearity, precision, trueness, sensitivity, recovery and matrix effects. The performances of both extraction methods were assessed in clinical samples obtained from patients treated with either apixaban or rivaroxaban. Additionally, we report on a patient with nonadherence to rivaroxaban treatment and fulminant pulmonary embolism.

Results: The method was linear from 2 to 500 ng/mL for all analytes, and quantification of DOACs was established within a run time of 2.0 min. Based on MS/MS analyte responses, relative matrix effects were better controlled for dabigatran after extraction with paramagnetic micro-particles. Internal standards fully compensated for recovery and matrix effects in all assays, yielding equivalent results for both methods. Apixaban and rivaroxaban concentrations determined in clinical samples after extraction with both methods were in good agreement ($R^2 = 0.990$).

Conclusions: A rapid and accurate multi-component UHPLC-MS/MS method for the quantification of four DOACs in human plasma was established. Paramagnetic micro-particles appear suitable for clean-up of plasma samples for LC-MS/MS-based therapeutic drug monitoring purposes.

Keywords: apixaban; dabigatran; edoxaban; LC-MS/MS; magnetic beads; rivaroxaban.

Introduction

For decades, vitamin K antagonists (VKAs) such as warfarin and phenprocoumon have served as oral anticoagulants to treat and prevent thromboembolic disease. VKAs are narrow therapeutic index drugs, characterized by large inter- and intra-individual variability of their dose-response relationship. These factors necessitate regular monitoring of the anticoagulant activity and frequent dose adjustments in patients treated with VKAs [1].

The direct thrombin inhibitor dabigatran and the direct factor Xa inhibitors apixaban, edoxaban and rivaroxaban have been developed and introduced as alternatives to VKAs [2]. Direct oral anticoagulants (DOACs) are indicated to prevent venous thromboembolism in patients after knee and hip replacement surgery and have also been shown to be effective to treat and prevent re-occurrence of deep vein thrombosis and pulmonary embolism. In Europe, rivaroxaban has also been approved for the prevention of atherothrombotic events in patients after an acute coronary syndrome with elevated cardiac biomarkers [3]. In patients with non-valvular atrial fibrillation and additional risk...
factors, DOACs have been shown to be at least as efficacious as warfarin for stroke prevention and proved to have safety advantages, in particular with respect to the risk of intracranial hemorrhage [4].

From both a physician and patient perspective, a significant benefit of DOACs is their ease of use, because these agents have been approved for use in fixed doses without routine coagulation monitoring [5]. Nevertheless, clinicians have to be aware of patient factors such as renal function, body weight and concomitant drugs that may require reduction of the prescribed DOAC dose [6, 7]. Clinical experience has led to discussions that knowledge of the anticoagulant activity is justified and adds to the safety profile of DOACs in special situations, such as use of DOACs in patients at extremes of body weight, assessment of nonadherence to medication, and before urgent surgery [3].

Conventional hemostasis tests (prothrombin time [PT], activated partial thromboplastin time [APTT], thrombin time [TT]) are differentially affected by DOACs and do not allow for precise monitoring of DOACs. Specific test assays, such as the calibrated diluted thrombin time and calibrated anti-Xa assays are available to determine plasma concentrations of DOACs based on factor IIa or factor Xa activity [8]. Mass spectrometry enables direct quantitation of analytes based on specific mass-to-charge ratios and is considered the gold standard for quantitative drug analysis [9, 10]. Liquid chromatography tandem mass spectrometry (LC-MS/MS) assays are advantageous, as they have a low limit of quantification and are less likely to suffer from reagent variability [11]. Several protocols for quantification of DOACs have already been developed and published [12]. However, some aspects need consideration when using LC-MS/MS for quantitative analysis. Undetected co-eluting matrix components may reduce or enhance the ionization efficiency of the analytes of interest, potentially affecting precision, trueness, and the limit of quantification (LOQ) of the assay [13–15]. Aside from matrix effects, extraction protocols based on protein precipitation with acetonitrile require centrifugation and are therefore hard to automate [16].

We previously developed a multi-analyte ultra-high performance liquid chromatography tandem mass (UHPLC-MS/MS) method for quantification of the three DOACs apixaban, dabigatran, and rivaroxaban in human plasma [17]. Here, we evaluated two independent extraction procedures and present a new and improved multi-analyte approach that enables simultaneous quantification of apixaban, dabigatran, edoxaban and rivaroxaban. Our routine extraction procedure based on protein precipitation with acetonitrile and subsequent centrifugation was compared to a protocol based on sample clean-up using paramagnetic micro-particles (here referred to as magnetic beads) and subsequent magnetic depletion that offers the potential for automated extraction.

Materials and methods

Materials

Pure substances of apixaban, dabigatran, edoxaban, and rivaroxaban were purchased from Molekula (Munich, Germany). [13C6]-apixaban, [13C6]-dabigatran, [13C7]-edoxaban, and [13C6]-rivaroxaban were obtained from Alsachim (Illkirch-Graffenstaden, France). The MagSiMUS-TDM® Type II kit containing the reconstitution buffer IS and the magnetic beads in suspension was kindly provided by MagnaMedics Diagnostics BV (Geleen, The Netherlands). A magnetic separator (MM-SepCore M12+12) was purchased from MagnaMedics Diagnostics BV. Commercial quality controls for apixaban and dabigatran were obtained from Technoclone GmbH (Vienna, Austria) and for rivaroxaban from CoaChrom Diagnostica GmbH (Maria Enzersdorf, Germany). Acetonitrile and formic acid in LC-MS standard quality were purchased from Merck (Darmstadt, Germany). Deionized water was purified with a Milli-Q Plus Ultrapure water system (Millipore Corporation, Bedford, MA, USA).

Stock solutions, calibration standards, and quality controls

Stock solutions of apixaban and rivaroxaban were prepared in acetonitrile and ultrapure water (70:30, v/v), of dabigatran in 0.1 M HCl, and of edoxaban in acetonitrile and 0.1 M HCl (50:50, v/v) and stored at −80 °C. For preparation of calibration standards (CS) and internal quality controls (IQC), working stocks containing all four DOACs at concentrations of 30.0 mg/L (CS) and 25.6 mg/L (IQC) were arranged. Citrated plasma (provided by the Department of Transfusion Medicine, University Hospital of Cologne) was spiked to obtain six CS (final concentrations of 2, 6, 20, 60, 200, and 500 ng/mL) and three IQC (5, 80, and 400 ng/mL). Different lots of blank plasma were used for CS and IQC. A mixture of all internal standards was prepared in acetonitrile for the routine extraction protocol and in acetonitrile and reconstitution buffer (proportion of volumes as recommended by the manufacturer) for the magnetic beads protocol (final concentrations of 100 ng/mL each).

Sample preparation

For all assays, samples were processed in parallel by means of routine extraction with acetonitrile and the MagSiMUS-TDM® Type II kit, and extracted samples analyzed in the same runs in mixed order. For routine extraction, 150 μL acetonitrile containing the internal standards were added to 50 μL plasma sample aliquots. The mixture was thoroughly vortexed, centrifuged (10 min, 4 °C, 15,000 g), and 120 μL of the clear supernatant subsequently transferred to LC-MS glass
vials (Macherey-Nagel, Düren, Germany). For sample clean-up with magnetic beads, 40 μL of magnetic beads suspension were added to 50 μL plasma sample aliquots and precipitation initiated by adding 150 μL of acetonitrile/reconstitution buffer containing the internal standards. After brief vortexing, samples were placed onto the magnetic separator (tube rack with integrated magnets) for 1 min. Subsequently, the clear extracts were carefully transferred to LC-MS glass vials. The principle of sample clean-up based on magnetic beads and subsequent magnetic purification is illustrated in Figure 1.

**Instrumentation and UHPLC-MS/MS conditions**

Chromatographic separation was performed on an Accela system (ThermoFisher Scientific, San Jose, CA, USA), equipped with an Accela 1250 pump and an Accela autosampler, fitted with a tempered tray and column oven. Tray temperature was set to 23 °C and the column oven maintained at 50 °C. A Hypersil Gold C8 column (50×2.1 mm, 1.9 μm particle size) served as stationary phase (ThermoFisher Scientific). Injection volume was set to 5 μL (partial loop injection). A gradient elution was used, and composition of the mobile phase consisting of acetonitrile (A) and 0.1% formic acid (B) (v/v) at a flow rate of 400 μL/min was as follows: 0.0–0.1 min 5% A, 0.1–0.2 min linear from 5 to 85% A, 0.2–1.2 min linear to 95% A, 1.2-1.4 min return to initial conditions and keep until 2.0 min.

UHPLC-MS/MS detection was carried out by a TSQ Vantage triple stage quadrupole mass spectrometer (ThermoFisher Scientific), operating in selected reaction monitoring (SRM) mode using heated positive electrospray ionization (HESI). Mass spectrometric parameters were: spray voltage 3 kV, vaporizer temperature 350 °C, and heated capillary temperature 300 °C. Nitrogen was used as sheath and auxiliary gas and set to 20 and 5 (arbitrary units). Argon was used as collision gas and pressure set to 1.5 mTorr. SRM transitions and respective collision energies were automatically optimized. TSQ Tune Master Software (version 2.3) was used for optimization of mass spectrometric conditions, and LCQuan software (version 2.6) for quantitative analysis and data evaluation.

**Method validation**

Method validation was carried out with a focus on linearity, precision, trueness, limit of detection (LOD) and quantification (LOQ), recovery, and matrix effects according to recommendations by US Food and Drug Administration (FDA) ‘Guidance for Industry – Bioanalytical Method Validation (Draft Guidance)’ and International Conference on Harmonisation (ICH) ‘Validation of Analytical Procedures’ [18, 19].

**Linearity:** For calibration, the peak area ratios of apixaban, dabigatran, edoxaban and rivaroxaban to internal standards were plotted against the concentration of the four DOACs (x-axis). Calibration curves were generated by least squares linear regression with a weighting factor of 1/x. Linearity was accepted if linear regression procedure gained R² > 0.99. A blank sample (blank plasma without internal standards) and a zero sample (blank plasma containing internal standards) were analyzed prior to each calibration.

**Precision and trueness:** Precision and trueness were assessed for all IQC levels as inter-day variability (over time on six different days) and intra-day variability (six replicates per concentration analyzed on 1 day). Precision was expressed as coefficient of variation (CV), calculated as the standard deviation of the observed concentrations divided by the mean concentration for each IQC level. Trueness was calculated as the deviation of the mean from the nominal concentration for each IQC level. Precision and trueness were accepted if the acceptance criterion ± 15% was not exceeded. Additionally, precision was assessed for commercial quality controls as inter-day variability (over time on six different days) and expressed as CV.

**Limit of detection, limit of quantification, and carry over:** LODs and LOQs were calculated from inter-day assay data, using the equations LOD = 3.3 σ/S′ and LOQ = 10 σ/S′, where σ is the standard deviation of the blank response and S′ is the slope of the calibration curve. Carry over was assessed by alternately injecting the highest calibration level solution for each IQC level. Carry over was calculated as the analyte peak area ratio of blank sample and the preceding calibration standard sample.

**Matrix effects and recovery:** Matrix effects and recovery were investigated according to Matuszewski et al. [16]. Briefly, three different matrices (A–C) were spiked with working stock solution to obtain DOACs concentrations at all IQC levels. Matrix A consisted of neat solution containing acetonitrile and 0.1% formic acid (85:15, v/v). For matrix B, six different lots of blank plasma were extracted with both the routine and magnetic beads protocol and then spiked with...
working stock solution (post-extraction spiking). To obtain matrix C, six different lots of blank plasma were spiked with working stock solution and then extracted with both protocols (pre-extraction spiking). All samples were subjected to UHPLC-MS/MS analysis and matrix effects and recovery calculated both from the observed analyte peak areas and analyte to internal standard ratios. Relative matrix effects were assessed by comparing MS/MS responses of analyte peak areas and analyte to internal standard ratios. Relative and matrix effects and recovery calculated both from the observed spiking. All samples were subjected to UHPLC-MS/MS analysis.

Retention times were 1.18 min for apixaban, 0.39 min for dabigatran, 1.07 min for edoxaban, and 1.20 min for rivaroxaban. Internal standards co-eluted with analytes. No significant interfering peaks were observed in different lots of extracted blank plasma matrices. The quality of chromatographic peaks obtained after extraction with both methods were comparable, except for edoxaban where chromatograms appeared more preferable after sample clean-up with magnetic beads. However, this difference did not influence edoxaban quantification. SRM transitions were (quantifier/qualifier): apixaban m/z 460.2→443.2/199.1; [13C6]-apixaban m/z 468.2→451.3/199.1; dabigatran m/z 472.2→289.1/144.1; [13C6]-dabigatran m/z 478.2→295.1/144.1; edoxaban m/z 548.2→366.3/152.2; [1H6]-edoxaban m/z 554.2→372.3/158.2; rivaroxaban m/z 436.1→144.9/231.1; [13C6]-rivaroxaban m/z 442.1→144.9/237.1.

Linearity, precision, and trueness

Both methods proved linear over the entire concentration range of 2–500 ng/mL with R2>0.99 for all analytes. Exemplary calibration curves are provided in Supplemental Figure 2. Inter- and intra-day precisions and trueness values remained well within the acceptance criteria and are summarized and compared in Table 1. Overall, similar results were obtained for both extraction methods. Mean DOAC concentrations and precisions obtained in analyses of commercial quality controls are presented in Supplemental Table 1.

Limit of quantification and carry over

The highest calculated LOQ was obtained for edoxaban at 2.01 ng/mL after routine extraction. The concentrations of the lowest CS were adjusted to the calculated LOQ of edoxaban for all analytes. Difference in sensitivity was most pronounced for apixaban where the calculated LOQ was 2-fold higher after clean-up with magnetic beads (Table 2). No carry over was observed (<20% of LOQ concentrations) except for dabigatran after routine extraction where a small carry-over of 0.13% of the response obtained in the preceding sample was determined. However, this effect did not relevantly impact quantification of dabigatran, and no further measures were applied.

Results

Liquid chromatography and mass spectrometry

Representative chromatograms of all DOACs after both extraction protocols are shown in Supplemental Figure 1. Retention times were 1.18 min for apixaban, 0.39 min for dabigatran, 1.07 min for edoxaban, and 1.20 min for rivaroxaban. Internal standards co-eluted with analytes. No significant interfering peaks were observed in different lots of extracted blank plasma matrices. The quality of chromatographic peaks obtained after extraction with both methods were comparable, except for edoxaban where chromatograms appeared more preferable after sample clean-up with magnetic beads. However, this difference did not influence edoxaban quantification. SRM transitions were (quantifier/qualifier): apixaban m/z 460.2→443.2/199.1; [13C6]-apixaban m/z 468.2→451.3/199.1; dabigatran m/z 472.2→289.1/144.1; [13C6]-dabigatran m/z 478.2→295.1/144.1; edoxaban m/z 548.2→366.3/152.2; [1H6]-edoxaban m/z 554.2→372.3/158.2; rivaroxaban m/z 436.1→144.9/231.1; [13C6]-rivaroxaban m/z 442.1→144.9/237.1.

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Matrix effects, recovery, and stability

Post-column infusion of the analytes during the UHPLC-MS/MS analysis of six different blank plasma showed ion
suppression for the factor Xa inhibitor transitions with maximum at 0.36 min and ion enhancement followed by suppression for dabigatran transition with maximum at 0.34 min after both routine and magnetic beads extraction (Figure 2). Quantitative assessment of matrix effects according to Matuszewski et al. [14] revealed that absolute matrix effects based on MS/MS analyte responses were lower for apixaban and edoxaban after magnetic beads extraction. Relative matrix effects observed between different lots of extracted blank plasma accounted for up to 44.6% variability of dabigatran MS/MS responses after routine extraction and were better controlled after clean-up with magnetic beads. Comparison of peak area ratios showed that isotopically labeled internal standards fully compensated for matrix effects in all assays and for both extraction protocols. These results are summarized in Table 3. Recoveries were satisfactory and ranged from 69.1% to 102.7% after routine extraction and from 84.2% to 103.2% after clean-up with magnetic beads. Internal standards compensated for recovery. Detailed results for recovery are provided in Supplemental Table 2.

Stability studies showed that all analytes were stable under all tested conditions. Except for edoxaban, where a decrease of concentration was found after 7 days of storage at room temperature for all IQC levels (69.4%–83.1% of the concentration found in fresh samples).

### Clinical application

Citrated plasma samples from patients treated with apixaban (n=10, male:female = 8:2) and from patients treated with rivaroxaban (n=30, male:female = 14:16) were collected. Patients with apixaban treatment were between 37 and 93 years old (mean 70.5 years) and received apixaban dosages between 5 and 10 mg/day. Patients treated with

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| *IQC, internal quality controls; *CV, coefficient of variation, calculated as SD/mean × 100; *calculated as mean/specified concentration ×100. |

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<th>Table 2: Calculated limits of quantification and detection.</th>
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rivaroxaban were between 40 and 89 years old (mean 68.3 years) and received rivaroxaban dosages between 10 and 30 mg/day, depending on indication and patient factors. The time of sampling was not standardized with respect to DOAC intake. Observed apixaban concentration ranges were 34.0–342.5 ng/mL after routine extraction and 29.5–345.7 ng/mL after clean-up with magnetic beads. In one patient sample, apixaban was found below the calculated LOQ. Observed rivaroxaban concentration ranges were 2.2–308.1 ng/mL after routine extraction and 3.1–293.1 ng/mL after extraction with magnetic beads. Concentration results were in good agreement ($R^2 = 0.990$; Figure 3).

Figure 2: Relative change of MS/MS signal intensities of apixaban (A), dabigatran (B), edoxaban (C), and rivaroxaban (D) during analysis of six different lots of blank plasma (post-column infusion experiment). Left: routine extraction; right: magnetic beads extraction. To visualize retention times of DOACs, respective chromatograms are laid behind.
Additionally, we report the case of a 69-year-old male patient who was admitted to our clinic due to acute dyspnea and chest tightness. In the emergency department, the patient presented with cardiogenic shock and respiratory insufficiency. Diagnostic CT pulmonary angiography confirmed diagnosis of central, bilateral fulminant pulmonary embolism. Due to progressive respiratory and hemodynamic instability, the patient underwent systemic thrombolytic therapy with alteplase. In the pre-hospital phase, the patient had received 5000 IU of unfractionated heparin, and parenteral anticoagulation was continued after successful thrombolytic therapy. Deep vein thrombosis of the left popliteal vein was confirmed by ultrasonography. The patient received polypharmacy (ramipril, torasemide, simvastatin, metformin, paroxetine, l-thyroxine, pantoprazole, and rivaroxaban) and was unable to recall why rivaroxaban was being prescribed. To assess exposure to rivaroxaban at the time of admission, a plasma sample was taken and subjected to LC-MS/MS analysis. The plasma concentration of rivaroxaban was found below the LOQ, consistent with non-adherence or inability to adhere to rivaroxaban treatment. During the course, parenteral anticoagulation was switched back to oral anticoagulant therapy with rivaroxaban (15 mg bid). To ascertain adequate exposure, rivaroxaban trough concentration was monitored after treatment initiation and found at 100.0 ng/mL and thus within the expected concentration range [20]. The patient was informed about the pharmacological properties of rivaroxaban and the importance of adherence.

Discussion

Clinical mass spectrometry is expansively used in laboratory medicine [21]. Owing to its high selectivity and sensitivity, mass spectrometry enables precise quantification of even
very low drug concentrations. Moreover, LC-MS/MS allows for the development of multi-component methods that can extend the spectrum of TDM service [22]. This technique also facilitates quantification of drugs such as DOACs in other matrices than plasma, e.g. in breast milk [23] or after prolonged transport or storage of a plasma specimen based on stability testing results. Due to its mode of detection, LC-MS/MS can be used to reliably quantify DOAC plasma concentrations in situations where compounds are present that can interfere with coagulation assays, such as heparins. In this situation, as illustrated in the clinical case, LC-MS/MS may enable differentiation between nonadherence to DOAC treatment and treatment failure. However, when employing LC-MS/MS in routine service, one has to keep in mind that results need to be made available 24/7. In this respect, calibrated coagulation assays remain a reasonable choice for many laboratories at present.

To our knowledge, only one method has been reported that describes simultaneous quantification of all four currently licensed DOACs by turbulent flow liquid chromatography with high-resolution mass spectrometry [24]. This method enables quantification within a total analysis time of 6 min over a linear calibration range of 1–500 ng/mL for all DOACs. While this method has advantages of its own, it certainly is more specialized compared to conventional LC-MS/MS. Since the introduction of DOACs, several LC-MS/MS methods have been reported for the quantification of a single DOAC or multiple DOACs in human plasma [12]. An important feature of our method is run time, which compared to our previously reported method [17] was further reduced from 2.5 to 2.0 min and which is shorter than run times in other methods enabling simultaneous quantification of at least three DOACs. A 4.75 min gradient run was reported by Schmitz et al. [25], while Baldelli et al. described a LC-MS/MS method with a total run time of 10 min [26]. Noguez and Ritchie reported an UPLC-MS/MS method for quantitation of apixaban, dabigatran, rivaroxaban, and warfarin within a run time of 5.5 min [27]. With respect to sensitivity, the calculated LOQs ranged from 0.84 to 2.01 ng/mL in the present study (Table 2) and were higher than those determined in our previous method (LOQs: 0.28–0.91 ng/mL). This can in part be explained by the sample dilutions, which were 1 in 4 and 1 in 4.8 according to the extraction protocols (compared to 1 in 3 in our previous study). Our method enables quantification of low and high DOAC concentrations as typical for LC-MS/MS methods, and quantitation was linear across the calibration range of 2–500 ng/mL for all DOACs. The method by Schmitz et al. was based on protein precipitation with acetonitrile, and calibration ranged from 23–750 ng/mL for apixaban, dabigatran and rivaroxaban. The calibration range reported by Baldelli et al. [26] was 1–500 ng/mL for these DOACs and the LOQs set to 1 ng/mL, while solid-phase extraction was applied prior to LC-MS/MS analysis. None-zero calibrators used by Noguez and Ritchie covered the concentration range from 5 to 1000 ng/mL, and samples were extracted with a methanol/hydrochloric acid solution.

Reference ranges for trough and/or peak DOAC plasma concentrations have not been established yet, and there is no rationale for routine monitoring of DOACs at present. However, a general understanding exists about the range of concentrations that can be expected on treatment. Although DOACs have a more predictable pharmacokinetic profile, even trough concentrations of DOACs may vary considerably between patients [28, 29]. For instance, in real-life patients with atrial fibrillation treated with rivaroxaban 15 or 20 mg od, median rivaroxaban plasma concentrations were 34 ng/mL (range 5–84 ng/mL) and 233 ng/mL (range 120–375 ng/mL) at trough and peak, respectively [30]. Likewise, median trough plasma apixaban concentrations in patients with atrial fibrillation were 48 ng/mL (range 15–83 ng/mL) and 77 ng/mL (range 29–186 ng/mL) for 2.5 mg bid and 5 mg bid dose groups, respectively [31]. Real-life concentration data for edoxaban are sparse at present. In an analysis of data from a phase 3 clinical trial evaluating edoxaban in patients with atrial fibrillation, median trough concentrations were 36.1 ng/mL (95% CI 24.2–48.0 ng/mL) in the 60 ng/day higher-dose group and 18.4 ng/mL (10.1–32.3 ng/mL, 95% CI) in the 30 ng/day lower-dose group [32]. In another study by Skeppholm et al., trough plasma concentrations of free (nonconjugated) dabigatran and total (including dabigatran glucuronides) dabigatran were measured in patients with atrial fibrillation. In all patients (i.e. 110 mg and 150 mg bid dose groups), the median free dabigatran concentration was 54 ng/mL (range 8–188 ng/mL) and the median total dabigatran concentration was 70 ng/mL (range 12–237 ng/mL) [33]. Peak dabigatran (nonconjugated) concentrations determined in atrial fibrillation patients 2–4 days post-treatment and 6–8 weeks after initiation of treatment with dabigatran 150 mg bid were 187±138 ng/mL (mean ± SD) and 159±83 ng/mL. Similarly, peak dabigatran concentrations in the 110 mg bid dose group were 153±61 ng/mL and 187±122 ng/mL [34]. In all of these studies, DOAC concentrations were determined by LC-MS/MS.

Care needs to be taken when interpreting UHPLC-MS/MS results for dabigatran. Dabigatran undergoes conjugation with activated glucuronide acid to yield pharmacologically active metabolites [35], and the active moiety of dabigatran will be underestimated when monitoring parent transitions, i.e. nonconjugated dabigatran, only [9, 25]. Schmitz et al. found that, on average, coagulation
assays such as the Hemoclot® calibrated diluted thrombin time assay yielded higher dabigatran concentrations compared to UHPLC-MS/MS [25]. For the determination of total, i.e. nonconjugated plus conjugated dabigatran, alkaline hydrolysis for 2 h and enzymatic cleavage by means of incubation with β-glucuronidase for 3 h have been reported [35, 36]. However, these approaches are not suited for a rapid LC-MS/MS assay. On the other hand, Schellings et al. described that commercial coagulation assays did not qualify to reliably measure trough levels of dabigatran, and only LC-MS/MS methods can currently be trusted in this regard. Yet, calibrated coagulation assays are suitable to exclude relevant dabigatran concentrations in emergency situations [37]. For edoxaban, the M4-metabolite contributes to the anticoagulant activity, but constitutes less than 10% of total edoxaban exposure [38, 39]. None of apixaban and rivaroxaban metabolites have been reported to contribute to their anticoagulant activities [40–42].

Analytical performance of LC-MS/MS methods may be impaired by ion suppression or enhancement effects caused by interfering matrix components, especially when dealing with complex matrices or multiple analytes [14]. Also, methods based on protein precipitation with acetonitrile and use of heated electrospray ionization were shown more susceptible for matrix effects compared to alternative sample pretreatment techniques or LC-MS/MS interfaces [13, 14]. In this respect, magnetic beads may differentially deplete interfering matrix compounds like salts, proteins and phospholipids compared to routine extraction with acetonitrile. Here, commercially available magnetic beads were evaluated, and previous reports employing this type of beads for clean-up of serum, whole-blood, and plasma matrices preceding LC-MS/MS analysis reported good control of matrix effects [16, 43, 44]. However, van den Bossche et al. found that the diminished matrix effect with magnetic beads was mainly due to dilution effects [16]. We aimed at reducing this potential bias by adjusting our routine extraction method, resulting in more comparable sample dilution (1 in 4 for routine extraction and 1 in 4.8 for magnetic beads protocol). Only subtle differences were seen in post-column infusion experiments between both extraction methods (Figure 2). In quantitative assessment, magnetic beads showed more preferable control of relative matrix effects between different lots of plasma based on MS/MS responses for dabigatran (Table 3). However, the stable isotope-labeled internal standards compensated for matrix effects in all assays, and as long as adequate internal standards are used for each analyte, this potential of magnetic beads likely will not be substantial. Rather, magnetic beads extraction may be advantageous with respect to control of matrix effects in multi-component assays without separate internal standardization for each analyte. Magnetic beads technology offers the potential for automation of the pre-analytical phase without centrifugation [16, 45]; however, this has not been evaluated in this study.

In summary, a rapid multi-analyte method for the simultaneous quantification of apixaban, dabigatran, edoxaban, and rivaroxaban in human plasma was developed. Apixaban and rivaroxaban concentrations determined in clinical samples after routine extraction with acetonitrile and sample clean-up with magnetic beads were in good agreement. The UHPLC-MS/MS method is suitable for TDM purposes, enabling rapid and accurate quantitation of very low and high plasma concentrations of all four currently licensed DOACs within one analysis. Apart from drug monitoring purposes in general or pharmacokinetic analyses, it can serve as a screening assay when it is not known if or which DOAC was taken.

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