Simultaneous quantification of direct oral anticoagulants currently used in anticoagulation therapy

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Abstract

Direct oral anticoagulants (DOACs) are among the most effective options to prevent serious thromboembolic events in patients with atrial fibrillation. Coagulation assays are used to assess DOAC activity, but lack the possibility to quantify drugs with concurrent pharmacodynamic effect. We developed a selective multi-drug assay to analyze apixaban, betrixaban, dabigatran, edoxaban, edoxaban M4, and rivaroxaban with ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC/MS/MS) in plasma fulfilling all requirements of the FDA und EMA guidelines for bioanalytical method validation. Plasma samples were extracted using solid phase extraction in a 96-well micro volume format. Chromatographic separation was performed on a Waters BEH Phenyl 1.7 μm column coupled to tandem mass spectrometry. Extraction recoveries exceeded 80%. Concentrations of 1–1000 ng/ml can be precisely quantified (correlation coefficient of >0.99) using 100 μL plasma volume. Intra-day and inter-day accuracies ranged between 91.0 % and 116 %. Precisions at low and high concentrations were below 13.3 %. The method was applied within a clinical drug trial and eight short pharmacokinetic profiles of patients under DOAC therapy were analyzed. The assay allows for highly sensitive and selective simultaneous quantification of DOACs in patient plasma samples.

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1. Introduction

Atrial fibrillation is a global disease with growing incidence and prevalence [1], associated with 20–30% of all ischemic strokes [2]. Anticoagulant drugs including direct oral anticoagulants (DOACs) are remarkably successful in avoiding embolic complications [3]. Due to their immediate inhibitor effect, DOACs have a rapid onset of action, which is, however, short-lasting because of terminal elimination half-lives that are considerably shorter than those of vitamin K antagonists. Therefore, to warrant adequate protection it is crucial that patients strictly adhere to therapy [4]. Adherence can be verified by concentration monitoring. Apart from adherence, monitoring DOAC concentrations has further advantages. As demonstrated for dabigatran and edoxaban, blood concentrations correlate well with the risk of bleeding and thromboembolism [5–7]. Monitoring can further support dose selection in complex clinical situations such as impaired renal function or polypharmacy with interacting co-medication [8,9].

Specific coagulation assays for DOAC concentration monitoring are already established in clinical practices [10], but none is specific enough to enable quantitative detection of all DOACs in a single assay. Because these assays require calibration to the individual compound, the specific DOAC taken by the patient must be known to allow reliable quantification. A number of liquid chromatography mass spectrometry (LC/MS) methods for a simultaneous and selective quantification of all DOACs have been reported [11–13], but they all lack the possibility of an easily applicable, fast, and sensitive quantification. In one assay, DOAC plasma samples prepared with protein precipitation were precisely but unconventionally quantified with high resolution orbitrap mass spectrometry [11]. Another method using the more common tandem mass spectrometry lacked sensitivity to reliably quantify trough concentrations (calibration range: 23–750 ng/ml) [12]. A third method finally used a sensitive
but less common and expensive method of paramagnetic beads for sample preparation [13]. We therefore aimed to establish one convenient protocol to analyze all DOACs in daily routine using low plasma volumes, solid phase extraction in 96-well format, and ultra-performance liquid chromatography tandem mass spectrometry (UPLC/MS/MS) for quantification. Therefore, this paper describes a specific and precise UPLC/MS/MS multi-drug assay to simultaneously quantify the currently approved DOACs apixaban, dabigatran, edoxaban, rivaroxaban, the active metabolite edoxaban M4, and the developing DOAC betrixaban in 100 µL plasma volume. The method was validated according to current guidelines of the Food and Drug Administration (FDA [14]) and European Medicines Agency (EMA [15]) and was applied to quantify short pharmacokinetic profiles of eight patients regularly treated with a DOAC.

2. Materials and methods

2.1. Chemicals and reagents

\(^{13}\text{C}_2\text{H}_8\)-apixaban, betrixaban, its internal standard (IS) \(^{13}\text{C}_6\)-betrixaban, dabigatran, \(^{13}\text{C}_6\)-dabigatran, edoxaban M4, rivaroxaban, and \(^{13}\text{C}_6\)-rivaroxaban were purchased from ALSACHIM (Illrich, France). Apixaban was obtained from BIOZOL (Eching, Germany). Edoxaban and \(^4\text{H}_8\)-edoxaban were kindly provided by Daiichi-Sankyo (Munich, Germany). Acetonitrile, methanol, and water (Biosolve, Valkenswaard, Netherlands) were of UPLC/MS grade. Ammonium formate (Sigma-Aldrich, Steinheim, Germany) was used to prepare the aqueous UPLC/MS/MS eluent. Sodium acetate buffer (pH=5.0; 0.2 M) used for solid phase extraction sample preparation was made by dissolving sodium acetate from Merck (Darmstadt, Germany) in water and adjusted to pH 5.0 with 100 % acetic acid from Roth (Karlsruhe, Germany). Analyte-free pooled human plasma for validation, calibration, and quality control samples was supplied by the local blood bank (IKTZ, Heidelberg, Germany).

2.2. Patient blood samples

Patient blood samples were collected in the course of a clinical study for analytical method comparison, which was approved by the responsible Ethics Committee of the Medical Faculty of Heidelberg University (S-575/2015). Written informed consent was obtained from all patients before participating in the study. Blood samples from patients under regular DOAC therapy were taken hourly over three hours after drug intake in lithium heparin tubes. Blood samples were immediately centrifuged (2000g, 10 min, 20 °C) and plasma was stored at −25 °C until analysis.

2.3. Calibrators, quality controls, and IS solution

Separate calibrator and internal quality control (QC) stock solutions were solved in acetonitrile/water and stored at −25 °C. Calibration working solutions were prepared at 1, 5, 10, 50, 150, 450, 750, and 1000 ng/mL by diluting the stock solutions with acetonitrile/water (1:1 v/v). QC working solutions were prepared at 3 (QC A), 500 (QC B), and 800 ng/mL (QC C) in acetonitrile/water (1:1 v/v). Calibration and QC working solutions were stored at −25 °C in light-protecting glass vials. Isotope-labeled IS were dissolved in acetonitrile/water (1:1 v/v) and aliquots of IS stock solutions were mixed to a working solution and stored at −25 °C.

2.4. Sample preparation

Calibrators and QCs were prepared by adding calibration or QC working solutions (25 µL) to blank plasma. For volume adjustment, acetonitrile/water (1:1 v/v; 25 µL) was added to every patient plasma sample (100 µL). Calibrators, QCs, and patient samples were spiked with 150 µL acetate buffer and 25 µL IS solution; the mixture was vortexed (5 s), and centrifuged (16,000g, 3 min) before solid phase extraction (SPE).

An aliquot of the supernatant (250 µL) was transferred onto µElution PRIME HLB 96-well cartridges (Waters, Eschborn, Germany) and slowly pushed through the cartridges using a 96-well positive pressure processor (Waters, Eschborn, Germany). Subsequently, wells were washed with adding methanol/water (5:95 v/v; 200 µL). After drying with nitrogen (20 psi, 30 s) the analytes were eluted with methanol (100 µL) into a 96-well sample collection plate (Waters, Eschborn, Germany). The eluates were evaporated to dryness under a stream of nitrogen (40 °C, 6.5 min; UltraTavPorvair Sciences Limited, Norfolk, UK). Residues were reconstituted in UPLC/MS/MS eluent, vortexed, and injected (20 µL) into the UPLC/MS/MS system.

2.5. Instrumental analysis

Analysis was performed on an Acquity UPLC system coupled to a TQD triple quadrupole mass spectrometer (Waters, Eschborn, Germany). The autosampler was kept at 15 °C. Separation was done on a 2.1 x 50 mm Acquity UPLC BEH Phenyl 1.7 µm column (Waters, Eschborn, Germany) at 40 °C. The eluents consisted of 95 % m/m ammonium formate in water with 5 % acetonitrile (A) and 100 % acetonitrile (B). The UPLC gradient separation was performed at a flow rate of 0.5 ml/min and started with 100 % A/0 % B (0.5 min). The ratio was changed to 5 % A/95 % B within 3.0 min (0.5–3.5 min) and kept until 4.0 min. Within the next 0.5 min, the system returned to its initial conditions. The analytes were positively ionized using heated electrospray ionization (Z-spray) with ionization parameters as follows: capillary voltage 1 kV, cone voltage 38 V, source temperature 150 °C, cone gas flow (N\(_2\)) 50 L h\(^{-1}\), desolvation gas flow (N\(_2\)) 850 L h\(^{-1}\), and desolvation temperature 500 °C. Mass analysis was performed by multiple reaction monitoring (MRM) using argon as collision gas. The ion transitions monitored were m/z 460.4 → m/z 443.2 for apixaban, m/z 469.5 → m/z 452.4 for \(^{13}\text{C}_2\text{H}_8\)-apixaban, m/z 452.2 → m/z 324.1 for betrixaban, m/z 458.3 → m/z 330.1 for \(^{13}\text{C}_6\)-betrixaban, m/z 472.4 → m/z 289.1 for dabigatran, m/z 478.4 → m/z 295.1 for \(^{13}\text{C}_6\)-dabigatran, m/z 548.4 → m/z 152.1 for edoxaban, m/z 521.3 → m/z 339.2 for edoxaban M4, m/z 554.5 → m/z 158.1 for \(^2\text{H}_8\)-edoxaban, m/z 436.2 → m/z 144.9 for rivaroxaban, and m/z 442.3 → m/z 144.8 for \(^{13}\text{C}_6\)-rivaroxaban. Peaks were integrated automatically by Masslynx 1.4 procedures. Batches with correlation coefficients r\(^2\) > 0.99 and accurately quantified QC concentrations (±15 %) were accepted.

2.6. Method validation

This multi-compound assay was validated according to FDA [14] and EMA guidelines [15]. Accuracy and precision were determined six-fold within three analytical batches at QC concentrations 1, 3, 500, and 800 ng/mL. Both parameters were evaluated within (intra) and between (inter) runs with an acceptance range of ± 15 % (except for LLOQ, 1 ng/mL: ± 20 %). Selectivity was tested by analyzing six blank human plasma samples from different sources. A possible effect of hyperlipemic or hemolytic plasma on accuracy was determined at 500 ng/mL. Stability tests were performed at QC levels (3, 500, and 800 ng/mL) in triplicate determinations. Long-term stability was tested for 1, 2, and 5 weeks at 4 °C and −25 °C. Benchtop stability (short-term stability) was assessed by analyzing QC plasma samples stored at 20 °C on a lab bench for 1 and 2 weeks. Carry-over was tested by analyzing blank eluent samples injected directly after the analysis of the highest calibration level. Recovery was determined at each QC level (3, 500, and 800 ng/mL) in
triplicate determinations by comparing peak areas from regularly spiked QCs with peak areas obtained from blank plasma spiked with QCs after sample preparation (representing a 100 % value). Matrix effects were analyzed for all analytes and IS in triplicate determinations at each QC level (3, 500, and 800 ng/ml). Peak areas of blank plasma spiked with analyte and IS after sample preparation

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**Table 1**

Intra-assay and inter-assay accuracies and precisions of all DOACs.

<table>
<thead>
<tr>
<th></th>
<th>Apixaban</th>
<th>Betrixaban</th>
<th>Dabigatran</th>
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<tr>
<td></td>
<td>LLOQ QC A QC B QC C</td>
<td>LLOQ QC A QC B QC C</td>
<td>LLOQ QC A QC B QC C</td>
</tr>
<tr>
<td><strong>Within batch</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>1 Mean [ng/ml]</strong></td>
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<td>1.1 3.1 509 811</td>
<td>1.0 2.8 474 763</td>
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<tr>
<td>Accuracy [%]</td>
<td>105 106 104 102</td>
<td>114 104 102 101</td>
<td>103 91.5 94.8 95.4</td>
</tr>
<tr>
<td>Precision [%]</td>
<td>1.7 2.0 0.8 0.9</td>
<td>0.6 0.5 1.2 1.0</td>
<td>3.1 2.2 0.8 0.6</td>
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<tr>
<td><strong>2 Mean [ng/ml]</strong></td>
<td>1.1 3.3 531 842</td>
<td>1.2 3.1 517 823</td>
<td>1.1 2.9 484 775</td>
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<tr>
<td>Accuracy [%]</td>
<td>111 109 106 105</td>
<td>115 105 103 103</td>
<td>107 96.8 96.9 96.8</td>
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<tr>
<td>Precision [%]</td>
<td>2.0 2.1 4.3 0.4</td>
<td>2.0 1.6 0.9 0.7</td>
<td>6.6 2.6 0.8 0.6</td>
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<td><strong>3 Mean [ng/ml]</strong></td>
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<td>1.2 3.1 519 827</td>
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<td>Accuracy [%]</td>
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<td>116 103 104 103</td>
<td>112 95.9 97.2 97.4</td>
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<tr>
<td>Precision [%]</td>
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<td>2.3 1.0 0.7 0.6</td>
<td>3.4 2.7 0.7 1.3</td>
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<tr>
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<tr>
<td>Accuracy [%]</td>
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<td>115 104 103 103</td>
<td>107 94.7 96.2 96.5</td>
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<tr>
<td>Precision [%]</td>
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<td>1.8 1.3 1.2 1.1</td>
<td>5.8 3.4 1.4 1.3</td>
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</tbody>
</table>

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**Fig. 1.** UPLC-/MS/MS chromatograms of a processed plasma sample at LLOQ level (1 ng/ml) with corresponding mass transitions and intensities of each analyte.
were compared with peak areas gained from pure eluent solution containing all analytes and IS at the respective QC concentrations. Normalized matrix factors were calculated by dividing the analytes’ matrix effects by IS’ matrix effects.

3. Results and discussion

3.1. Chromatography and mass spectrometry

After preliminary tests with different columns (Acquity UPLC BEH C18, CSH C18, Waters, Eschborn, Germany) and eluents (e.g. 0.01 % aqueous formic acid solution), the best separation and most intense [M+H]+ ions were achieved on a phenyl UPLC column with a gradient program composed of an aqueous 5 mM ammonium formate solution and acetonitrile. Fast and sharp peaks with peak width at baseline of 3–6 s depending on the analyte were achieved (Figs. 1–3). With a cycle time of 100 ms our method resulted in at least 20 data points per peak.

3.2. Sample preparation

A routine monitoring assay requiring only minimal plasma volume (100 µL) and offering the possibility of automation is certainly favorable. Different from an earlier method using paramagnetic micro-particles for sample extraction [13], we chose µElution SPE which is more convenient and can easily be scaled up to automation. Triplet determinations of recovery rates for all analytes and their internal standards at QC A to C level showed that the

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**Table 2**

<table>
<thead>
<tr>
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<td>QC B [%]</td>
<td>QC C [%]</td>
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<td></td>
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<td>−0.9</td>
<td>−2.5</td>
<td>2.2</td>
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<td>Dabigatran</td>
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<td>−11.0</td>
<td>−8.7</td>
</tr>
<tr>
<td>Edoxaban</td>
<td>0.9</td>
<td>−1.0</td>
<td>−1.8</td>
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<tr>
<td>Edoxaban M4</td>
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<td>−10.1</td>
<td>−5.5</td>
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<tr>
<td>Rivaroxaban</td>
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<td>−3.9</td>
<td>−1.8</td>
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<tr>
<td><strong>+4 °C</strong></td>
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<tr>
<td>Apixaban</td>
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<td>7.6</td>
<td>5.4</td>
</tr>
<tr>
<td>Betrixaban</td>
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<td>−1.5</td>
<td>−3.4</td>
</tr>
<tr>
<td>Dabigatran</td>
<td>−9.3</td>
<td>−7.8</td>
<td>−9.8</td>
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<tr>
<td>Edoxaban</td>
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<td>1.0</td>
<td>0.8</td>
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<td>−15.2</td>
<td>−19.6</td>
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<td>−4.9</td>
<td>−4.7</td>
</tr>
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<td>−21.9</td>
<td>−23.2</td>
</tr>
<tr>
<td>Rivaroxaban</td>
<td>−4.3</td>
<td>−5.6</td>
<td>−3.9</td>
</tr>
</tbody>
</table>

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**Fig. 2.** UPLC/MS/MS chromatograms of plasma samples at 500 ng/ml (processed QC B sample) with corresponding mass transitions and intensities of each analyte.
used hydrophilic-lipophilic material retained all analytes effectively. Good recovery rates from plasma were achieved and ranged between 85.5 % to 93.1 % for apixaban and $^{13}$C$_2$H$_8$-apixaban, 109.7 % to 120.1 % for betrixaban and $^{13}$C$_6$-betrixaban, 80.6 % to 84.8 % for dabigatran and $^{13}$C$_6$-dabigatran, 98.9 % to 100.9 % for edoxaban and $^2$H$_8$-edoxaban, 83.9 % to 86.8 % for edoxaban M4, and 101.6 % to 104.2 % for rivaroxaban and $^{13}$C$_6$-rivaroxaban. Results from matrix effect investigations revealed that the used sample preparation protocol effectively removed co-eluting biological compounds. Acceptable ion suppression was present for betrixaban (87.9 %).
whereas all other analytes were subject to slight ion enhancement (apixaban: 112.0 %, dabigatran: 101.9 %, edoxaban: 114.2 %, and rivaroxaban: 108.3 %). With the use of an isotope-labeled internal standard this effect is controlled as shown in normalized matrix factor values ranging between 0.94 (apixaban, edoxaban), 0.98 (dabigatran), 1.01 (betrixaban), and 1.02 (rivaroxaban). Edoxaban M4 showed reproducible, but concentration-dependent matrix effects. At QC A (3 ng/ml) ion enhancement was present (124.9 %), whereas at higher concentrations (500 and 800 ng/ml) ionization was suppressed (86.0 %). These varying matrix effects do not affect the quantification of edoxaban M4, as proven by valid quality controls analyzed within each batch.

3.3. Method validation

The combined protocol using solid phase extraction and UPLC/MS/MS resulted in a method fulfilling all FDA and EMA standards for bioanalytical method validation [14,15]. Calibration ranging between 1 and 1000 ng/ml resulted in good correlation coefficients ($r^2$) of $\geq 0.9988$ (apixaban), $\geq 0.9991$ (betrixaban), $\geq 0.9975$ (dabigatran), $\geq 0.9983$ (edoxaban), $\geq 0.9968$ (edoxaban M4), and $\geq 0.9935$ (rivaroxaban). Inter-assay accuracies (QC A-C) ranged from 91.0 % to 113 % and intra-assay accuracies (QC A-C) varied between 94.7 % and 112 %. A maximum inter-assay and intra-assay precision deviation of 8.9 % and 6.5 % was present respectively. Accuracies at LLOQ level were between 91.6 % and 116 % for all analytes (Table 1). Similar to other multi-drug assays published before, DOACs can be analyzed down to the ng/ml range [11–13]. With a maximum precision deviation of 13.3 % (Table 1), our assay allows a precise quantification of at least 1 ng/ml (LLOQ) for apixaban, betrixaban, dabigatran, edoxaban, edoxaban M4, and rivaroxaban. Selectivity was confirmed by the absence of interfering peaks in six different lots of pooled human blank plasma donated by healthy volunteers. Samples spiked into hyperlipemic and hemolytic plasma showed acceptable accuracies for all DOACs ranging between 86.2–105 % and 94.0–104 %, respectively, proving that the assay performance is also valid for hyperlipemic and hemolytic plasma samples. Chromatograms of a spiked plasma sample at LLOQ level (Fig. 1), at QC B level (Fig. 2), and a human plasma sample for each DOAC (Fig. 3) are depicted in Figs. 1–3. Stability was tested at lower (3 ng/ml), medium (500 ng/ml), and high (800 ng/ml) QC concentration and revealed good long-term and short-term stability for all analytes except edoxaban and its metabolite. In accordance with earlier publications [11,13], both were unstable when stored at temperatures above $-25 \, ^\circ\mathrm{C}$ for 5 weeks (Table 2). If the analyte is unknown and samples must be stored for more than one week, we thus recommend freezing plasma DOAC samples at $\leq -25 \, ^\circ\mathrm{C}$. Likely due to its high response to the TOQ mass spectrometer, slight carry-over was observed for betrixaban, which makes it necessary to inject a blank sample between patient samples to reduce carry-over. Other DOACs showed no carry-over.

3.4. Method application to patient samples

Short pharmacokinetic profiles in eight patients regularly treated with apixaban, dabigatran, edoxaban, and rivaroxaban are shown in Fig. 4. Therapeutic ranges for DOACs have not been defined by any manufacturer, but plasma concentrations under steady state conditions have been reported from Phase III trials [5,7,16,17]. The quantified concentrations are comparable to published values, suggesting that our method qualifies to monitor patient plasma samples.

3.5. Limitations

With focus on future prospects, if DOAC monitoring becomes a routine analysis, an external quality control system with real patient samples should be introduced. An external quality control system (round robin tests) would guarantee that every laboratory reliably quantifies DOACs in patient samples for a better comparability between the laboratories. A limitation of this assay is that the turnaround time is about two hours from sample receipt to result reporting and it lacks to quantify the active metabolites of dabigatran (dabigatran acylglucuronides).

4. Conclusions

Multi-drug assays offer the possibility to analyze different DOAC samples in parallel, which saves time and cost. So far this is an advantage of mass spectrometry, since coagulation assays do not distinguish between drugs revealing identical pharmacodynamic effects and have to be calibrated to the respective compound making them difficult to use in unconscious patients with unknown drug histories. A number of mass spectrometry methods for the quantification of DOACs have been published in the past, but only few were multi-drug assays [11–13] and to our knowledge none includes betrixaban, which was recently FDA approved and is currently under EMA investigation for market approval. By using μElution SPE, a LLOQ of 1 ng/ml was reached, which allows reliable quantification of plasma trough concentrations present under therapeutic DOAC maintenance doses. Furthermore, μElution SPE is convenient and can be scaled up to automated SPE methods, which makes our method of advantage if it should be established for routine measurements.

Conflict of interest statement

JB, KIF, AH, and LT, report no financial or personal relationships that could potentially be perceived as influencing the research described herein.

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References

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