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Measurement of apixaban, dabigatran, edoxaban and rivaroxaban in human plasma using automated online solid-phase extraction combined with ultra-performance liquid chromatography-tandem mass spectrometry and its comparison with coagulation assays

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ABSTRACT

Background: Measurement of direct oral anticoagulants (DOACs) concentration in patient blood is essential in special clinical circumstances.
Methods: We developed a fast, selective and sensitive method for simultaneous measurement of DOACs in human plasma consisting of an automated online solid-phase extraction method coupled with ultra-performance liquid chromatography electrospray ionization-tandem mass spectrometry (online SPE-UPLC-MS/MS).
Results: The calibration curves of all DOACs were linear over the working range (apixaban: 0.25–760 µg/L, $r > 0.99$; dabigatran: 0.5–900 µg/L, $r > 0.99$; edoxaban: 0.6–800 µg/L, $r > 0.99$; rivaroxaban: 0.5–900 µg/L, $r > 0.99$). Limits of detection in the plasma matrix were < 0.2 µg/L, whereas the lower limits of quantification were < 0.6 µg/L for all DOACs. The intraassay and interassay CV for all DOACs were < 6%. Mean recoveries were between 61.4% and 91.6%. Method comparison between our online SPE-UPLC-MS/MS assay and commercially available functional based coagulation assays using patient samples showed a high degree of correlation for all investigated DOACs.
Conclusions: We developed and validated the first online SPE-UPLC-MS/MS method for fast, sensitive, specific, and reliable measurement of the new generation of DOACs and compared this method with commercial available coagulation assays.

1. Introduction

The direct oral anticoagulants (DOACs) [1] dabigatran etexilate (Pradaxa®, Boehringer-IngelheimPharma GmbH & Co. KG), apixaban (Eliquis®, Bristol-Myers Squibb/Pfizer), edoxaban (Savaysa®, Daiichi Sankyo Inc.), and rivaroxaban (Xarelto® - Janssen and Bayer HealthCare) are licensed in the United States (US), the European Union (EU), and many other countries worldwide for prevention of stroke and systemic embolism in patients with non-valvular atrial fibrillation, treatment and secondary prevention of venous thromboembolism (VTE), and prevention of VTE after major orthopedic surgery [2]. Further clinical indications for DOACs include thromboprophylaxis in hip or knee replacement surgery [3]. Dabigatran is a reversible direct thrombin inhibitor, whereas the "**–*xa*ban**" drugs apixaban, edoxaban and rivaroxaban are reversible direct factor **Xa** inhibitors [4]. DOACs are small molecules which bind directly to the active side of pivotal procoagulant proteases. They do not require routine monitoring because pharmacokinetic and pharmacodynamic responses are reliably predicted in patients with sufficient renal function who are not taking other interacting drugs [5]. However, there will be clinical circumstances in specific patients when laboratory measurement of these drugs may be warranted. The knowledge of drug levels of specific patients may be important in conjunction with bleeding or thromboembolic events, suspected non-compliance or overdose, invasive

Abbreviations: aPTT, activated partial thromboplastin time; BEH, bridged ethyl hybrid; CV, coefficient of variation; DOACs, direct oral anticoagulants; DTI, direct thrombin inhibitor(s); IS, internal standard; MRM, multiple reaction monitoring mode; MS/MS, tandem mass spectrometry; LOD, limit of detection; LLOQ, lower limit of quantification; POCT, point-of-care-testing; PT, prothrombin time; RT, room temperature; SPE, solid-phase extraction; UPLC, ultra-performance liquid chromatography; VTE, venous thromboembolism

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procedures, patients taking other drugs that are known to significantly affect pharmacokinetics or patients with deteriorating renal function [3–5].

Even if highly DOAC-reactive reagents are used, normal results of global coagulation tests (aPTT and PT) are not suited to guide emergency treatment [6]. There are a few situations where exclusion diagnostics could be performed with point-of-care-testing (POCT), taking into account the high limitations of the method [7]. When a POCT test is used to exclude DOAC medication, patient safety is paramount. Accordingly, a safe cut-off should be used for the POCT analysis. As a result, many patients with a DOAC concentration below the required level (e.g. < 30 ng/mL for surgical procedures) are identified as being false-positive [8]. In the current literature, the use of a specific test (aXa and aIIa based tests) or mass spectrometric analysis is recommended [9]. Coagulation-based assays are unsuitable for measuring a specific drug concentration if the sample contains more than one anticoagulant, e.g. in samples with heparin and apixaban or rivaroxaban [10]. Under specific circumstances like high rivaroxaban concentrations, measurements of dabigatran using automated Direct Thrombin Inhibitor assay (DTI assay) could be influenced [11]. In principle, the highly specific and sensitive measurement of DOACs by LC-MS/MS is the method of choice (gold standard). Recently, several LC-MS/MS methods to measure these new DOACs were developed [11–21]. Furthermore, most of the previously mentioned procedures describe the measurement of only one [17], two [12,15], or three DOACs [13,16,18,19], whereas only three LC-MS/MS methods [14,20,21] were developed to measure all four currently licensed DOACs. One of these methods used turbulent flow liquid chromatography with high-resolution mass spectrometry [14] and the other two methods used ultra-performance liquid chromatography coupled to tandem mass spectrometry [20,21] for the determination of the DOACs. However, none of these previously described LC-MS/MS methods [14,20,21] for the simultaneous measurement of all four currently licensed DOACs used automated online solid-phase extraction for sample preparation to obtain high-purity samples which prevent mass spectrometer contaminations, and to increase the measuring sensitivity and selectivity. A comparison between the advantages and disadvantages of most of these new methods could be found in our recently published work on this subject [11]. Here, we develop an improved method to measure apixaban, dabigatran, edoxaban, as well as rivaroxaban, simultaneously using an automated online capturing act onto a trapping column for an additional sample preparation after protein precipitation. After this act the analytes of the captured sample were back transferred and the analytes were quantified using ultra-performance liquid chromatography electrospray ionization-tandem mass spectrometry. In addition, we compared the apixaban, dabigatran, edoxaban, and rivaroxaban concentration of patient samples measured by commercially available automated functional based assays with the direct measurement of the drugs in the samples using our LC-MS/MS assay.

2. Materials and methods

2.1. Reagents, internal standards, calibrators, and quality-control materials

Apixaban and edoxaban were obtained from Toronto Research Chemicals (North York, Canada). [^{13}C,$^{2}H_7$]-apixaban, dabigatran, [$^{13}C_6$]-dabigatran, [$^{2}H_6$]-edoxaban, rivaroxaban, and [$^{13}C_6$]-rivaroxaban were purchased from Alsachim (Strasbourg, France). More details are presented in Supplementary material.

2.2. Plasma samples

The plasma samples were collected in accordance with the German Act on Medical Devices (MPG, guideline 98/79/EG) for the collection of human residual material to evaluate suitability of an in vitro diagnostic medical device (§24). Hence, there was no need for an ethical approval as all materials used in this study were waste from routine laboratory diagnostics. More details are presented in Supplementary material.

2.3. Sample preparation

Sample preparation was performed in a 1.5-mL polypropylene microcentrifuge tube. 100 µL each of citrate plasma sample, calibrator or quality-control sample was added to 200 µL 0.1 M $ZnSO_4$ solution. After 700 µL IS solution (see above; further information can be found in Supplementary material) was added, the mixture was vortex-mixed for 5 s, centrifuged at 14,000 x *g* at RT for 5 min, and thereafter, 500 µL of the clear, colorless supernatant was transferred to the autosampler vessel.

2.4. Online SPE-UPLC-MS/MS measurements of DOACs

Instrument setup is shown in Fig. S1. The 2D UPLC system (Waters Acquity UPLC H-class with 2D Technology System) consists of an UPLC quaternary solvent manager for the 1st dimension (pump 1) and an UPLC binary manager for the 2nd dimension (pump 2), a six-port 2-position valve, column oven, and autosampler. The hardware configuration included a Waters Xevo TQ-S triple quadrupole mass spectrometer (Waters, Milford, MA) fitted with a Z Spray ion source. The automated sample extraction was performed using a 2.1 × 30-mm reverse phase cartridge (column 1, Waters, XBridge C8, 10 µm) followed by an analytical separation which was performed on a 2.1 × 50-mm reverse phase column (column 2, Waters, Acquity UPLC BEH C18, 1.7 µm). The chromatographic conditions, as well as the chromatographic gradients are presented in Table 1A-C. Column 1 was equilibrated with 35% methanol (*v*/v) containing 0.1% formic acid (v/v) and 2 mmol/L ammonium acetate (Table 1A, Fig. S1A). Thereafter, a 1 µL sample was injected at a flow rate of 0.1 mL min^{-1}. After enrichment of analytes and IS on column 1 at 0.40 min, the valve was switched to position 2 (Table 1B, Fig. S1B), the back-flush elution was performed by the mobile phase, and the eluate was separated on the analytical column (column 2) according to the gradient presented in Table 1C (Fig. S1B).

Table 1
A: Chromatographic conditions of the extraction column for the analysis of apixaban, dabigatran, edoxaban, and rivaroxaban

Step	Time (min)	Flow rate (mL min^{-1})	Solvent A (%)	Solvent B (%)	Gradient
1	uninterrupted	0.1	65	35	isocratic

B: Valve actuation.

Step	Time (min)	Event	Action
1	0.40	Valve switching	Position 2
2	3.00	Valve switching	Position 1

C: Back-flush UPLC gradient elution program for the analysis of apixaban, dabigatran, edoxaban, and rivaroxaban.

Step	Time (min)	Flow rate (mL min^{-1})	Solvent A (%)	Solvent B (%)	Gradient
1	initial	0.28	1	99	isocratic
2	0.1	0.28	1	99	isocratic
3	0.6	0.28	65	35	isocratic
4	2.4	0.28	30	70	linear
5	2.7	0.28	30	70	isocratic
6	2.9	0.28	1	99	isocratic

Solvent A, 0.1% formic acid in water containing 2 mmol/L ammonium acetate; Solvent B, 0.1% formic acid in methanol containing 2 mmol/L ammonium acetate.