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Determination of dabigatran, rivaroxaban and apixaban using UPLC-MS/MS and comparison with coagulation assays for therapy monitoring

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Recently, three novel oral anticoagulants (NOACs) have been registered by the FDA and European Commission for prevention of deep vein thrombosis in patients undergoing knee or hip replacement surgery and for prevention of stroke in patients with non-valvular atrial fibrillation (AF): dabigatran, rivaroxaban and apixaban (1). In December 2012, reimbursement for dabigatran and rivaroxaban was arranged for AF patients in the Netherlands (2). NOACs are direct inhibitors of coagulation factors; dabigatran inhibits thrombin and rivaroxaban and apixaban inhibit factor Xa. The pharmacokinetics and pharmacodynamics of these new drugs are more predictable than those of the traditionally used coumarin-derived anticoagulants. Furthermore, less food and drug-drug interactions have been reported (3). It was therefore advocated that monitoring would be obsolete (4). However, recent studies have shown that laboratory monitoring of NOACs is important, e.g. in patients having deviating posture, diminished renal function or in emergency (bleeding) situations (5). Many new coagulation assays have been developed, because conventional assays are typically not suitable for quantification (6). At this moment, it is unclear which coagulation assays are most suited for the measurement of NOACs. Our goal is therefore the development and validation of a UPLC-MS/MS method for the quantification of dabigatran, rivaroxaban and apixaban, to serve as a reference technique for comparison with several coagulation assays.

Methods

UPLC-MS/MS

Plasma and full blood were spiked with dabigatran, rivaroxaban and apixaban for a 6-point calibration (23-750 ng/mL) and quality control (QC) (50, 275, 500 ng/mL). Isotopically labelled internal standards (^{13}C and ^{2}H) of the NOACs were added, followed by sample clean-up using protein precipitation. Analysis was done with UPLC-MS/MS using a 4.75 minute, two-step Multiple Reaction Monitoring (MRM) method. Validation of the method was done by determining specificity, plasma matrix effects, accuracy and total precision (25 samples per QC level), lower limit of detection (LLOD, signal-to-noise-ratio (SNR)=3), lower limit of quantification (LLOQ, SNR=10) carry-over, recovery, stability (3x freeze-thaw, plasma and prepared sample storage at 4˚C and 20˚C) and possible interferences for hemolysis, lipemia and icterus. The detection of dabigatran acylglucuronide, the major dabigatran metabolite, was added to the MRM method. Analysis of acylglucuronide hydrolysis was performed by incubation of random patient samples with NaOH for two hours (7).

Coagulation assays

Several coagulation assays were validated by ten-fold calibration, total precision and accuracy determination (20 samples per QC level). The dabigatran coagulation assays tested were Pefakit PiCT (Pentapharm), ECA-T (Stago), Hemoclot dTT (Hyphen) and Stago dTT (in-house modified Stago method). The coagulation assays tested for rivaroxaban and apixaban were Pefakit PiCT (Pentapharm) and Liquid anti-Xa (Stago). Plasma was spiked with dabigatran, rivaroxaban or apixaban for calibration (750 ng/mL) and QC (100 and 400 ng/mL for dabigatran; 50 and 300 ng/mL for rivaroxaban and apixaban). In-house calibrators were used for all tests, except for the Hemoclot dTT assay. Stability of dabigatran and rivaroxaban spiked plasma was tested by frequent analysis of the samples by the Stago dTT and Liquid anti-Xa assay for 48 hours. Trueness of the four coagulation assays for quantification of dabigatran was evaluated by comparison with UPLC-MS/MS analysis using 37 random anonimized samples, obtained with permission from patients using dabigatran.

Results and discussion

UPLC-MS/MS

Calibration lines for all three NOACs were excellent (R^2=0.99). No significant differences could be seen between plasma and full blood calibration lines, indicating that the studied NOACs are not adsorbed to erythrocytes in vitro and that spiked citrated plasma can be used for analysis. Additionally, no bias was found in spiked hemolytic, icteric and lipemic plasma. Stability tests showed adequate stability during three

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freeze-thaw cycles, 24-hours plasma storage at 20˚C and 8-day sample storage at 4˚C. Specificity of the UPLC-MS/MS was good and LLOD and LLOQ were <1 ng/mL for all three NOACs. Matrix effects and carry-over were absent. For dabigatran, rivaroxaban and apixaban, the relative recovery was 73%, 78% and 104%, respectively. This was considered acceptable, due to the addition of the internal standards. Acceptance criteria for accuracy and precision were applied as given by the FDA (100±15% and <15%, respectively) (8). Precision data of the UPLC-MS/MS method were good; accuracy was only inadequate for low concentration rivaroxaban (see table 1). This might be explained by the small amounts of NOAC used to spike the citrated plasma.

The estimated mean amount of dabigatran acylglucuronide was 12% (range 0-45%) of the measured dabigatran concentration. No dabigatran acylglucuronide could be detected in the MRM-tracing after NaOH-incubation, indicating that the glucuronides were indeed fully hydrolyzed from dabigatran.

Coagulation assays

After calibration of the coagulation assays, precision and accuracy were determined using QC samples (see table 1). Accuracy and precision boundaries were applied as specified by the FDA (8). Reproducibility of the in-house modified Stago dTT method was superior compared to the Hemoclot dTT assay. The ECA-T test showed adequate precision and agreement at high concentrations, but a bias at lower concentrations. Therefore, the fitted model from the calibration should be reconsidered for assay improvement. The PiCT assay showed poor accuracy at low concentrations. For rivaroxaban, the PiCT assay showed inadequate precision, while the Liquid anti-Xa assay showed unacceptable accuracy for the high QC level. For apixaban a reproducible PiCT assay could not be developed, the Liquid anti-Xa assay showed unacceptable precision. Taking precision, accuracy and assay stability into account, the preferred assays are the Stago dTT for dabigatran and the Liquid anti-Xa assay for rivaroxaban and apixaban.

Stability testing of plasma containing dabigatran or rivaroxaban showed that plasma can be stored for at least 48 hours at room temperature before analysis.

Comparison of the different coagulation assays for dabigatran with the UPLC-MS/MS method in random patient samples (see figure 1) showed the best correlation and agreement for the Stago dTT assay. The PiCT assay, and in lesser extent the Hemoclot dTT assay, showed an unacceptable scatter. The ECA-T assay again showed a bias in the low concentration range.

Table 1. Mean concentration (C mean), total (inter + intra-assay) precision and accuracy data for the UPLC-MS/MS method (25 samples per QC level, measured over 5 days) and coagulation assays (20 samples per QC level, measured over 10 days). Expected concentrations: 50, 275 and 500 ng/mL for UPLC-MS/MS, 100 and 400 ng/mL for dabigatran coagulation assays and 50 and 300 ng/mL for rivaroxaban and apixaban coagulation assays.

	C mean			Total precision			Accuracy		
	level 1 (ng/mL)	level 2 (ng/mL)	level 3 (ng/mL)	level 1 (%)	level 2 (%)	level 3 (%)	level 1 (%)	level 2 (%)	level 3 (%)
UPLC-MS/MS									
Dabigatran	52.8	283.5	504.6	6.5	3.9	4.6	105.5	103.1	100.9
Rivaroxaban	58.3	314.0	568.6	7.6	8.3	8.5	116.5	114.2	113.7
Apixaban	47.3	253.1	457.1	10.1	9.8	8.4	94.6	92.0	91.4
Coagulation assays dabigatran									
Hemoclot dTT	115.6	490.8		33.5	35.1		125.2	131.0	
Stago dTT	99.2	418.5		10.3	8.0		107.4	111.7	
ECA-T	77.4	405.7		16.7	6.0		83.9	108.3	
PiCT	155.7	372.0		17.1	11.0		168.6	99.3	
Coagulation assays rivaroxaban									
PiCT	65.1	313.6		14.1	10.2		79.6	101.8	
Liquid anti-Xa	87.2	367.7		5.2	3.8		106.7	119.4	
Coagulation assays apixaban									
Liquid anti-Xa	89.3	313.5		18.6	15.4		103.1	93.2	

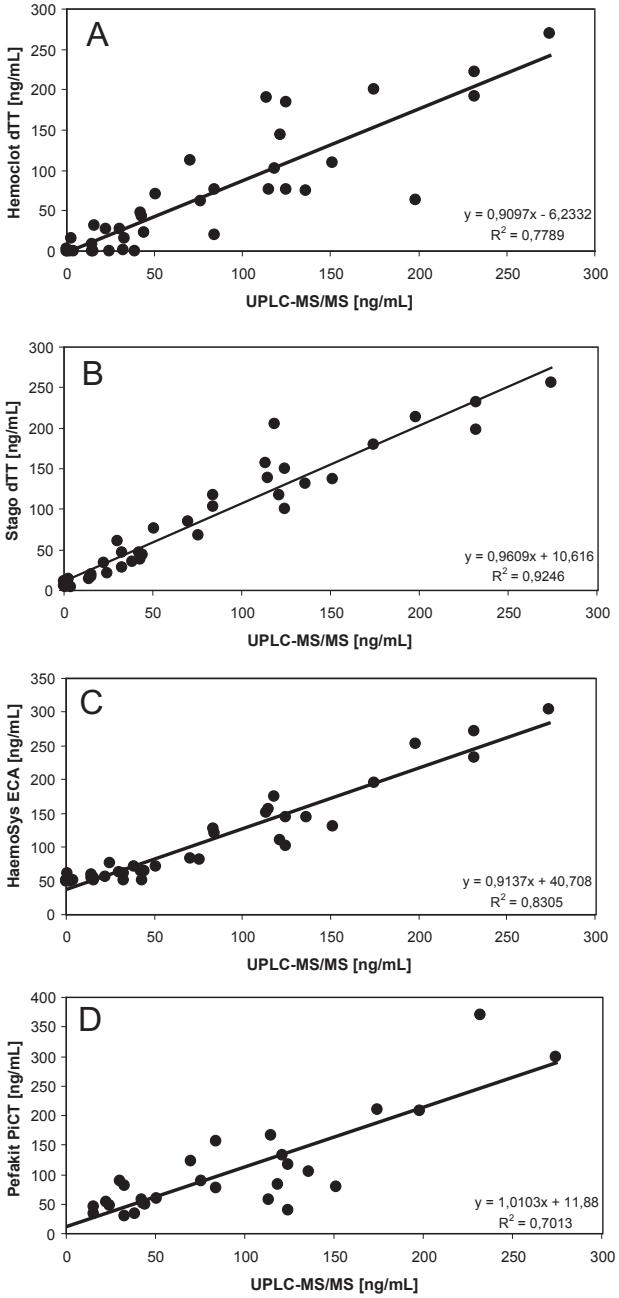

Figure 1. Correlation and linear regression of dabigatran coagulation assays with UPLC-MS/MS in random patient samples (n=37): Hemoclot dTT (A), Stago dTT (B), ECA-T (C) and Pefakit PiCT (D).

Conclusions

A UPLC-MS/MS method for quantification of dabigatran, rivaroxaban and apixaban was successfully developed and validated. Dabigatran's major metabolite, dabigatran acylglucuronide, could also be determined using UPLC-MS/MS. Based on our reproducibility study and patient results the superior coagulation assay for dabigatran is the Stago dTT assay. For rivaroxaban and apixaban, the preferred coagulation test is the Liquid anti-Xa assay.

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