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Short communication

Development of a fast and simple liquid chromatography–tandem mass spectrometry method for the quantitation of argatroban in patient plasma samples

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ABSTRACT

An ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) method for the direct measurement of argatroban in human plasma was developed and compared with the activity-based Hemoclot Thrombin Inhibitors assay. UPLC–MS/MS was performed using diclofenac as an internal standard. In summary, argatroban and diclofenac were extracted from 100 μL of plasma using a methanol precipitation protocol, and chromatographic separation was performed on an ACQUITY™ TQD mass spectrometer using a UPLC C18 BEH 1.7 μm column with a water and methanol gradient containing 0.1% formic acid. The detection and quantitation were performed using positive ion electrospray ionization and multiple reaction monitoring (MRM) mode. The UPLC–MS/MS method was linear over the concentration range of 0.003–3.0 μg/mL, with a lower limit of quantitation for argatroban of 0.003 μg/mL. The intra- and inter-assay imprecision was less than 12% at the plasma argatroban concentrations tested. Good correlation was demonstrated between the UPLC–MS/MS method and the indirect activity-based assay for determination of argatroban. However, increased plasma fibrinogen levels caused underestimation of argatroban levels using the indirect activity-based assay, whereas the UPLC–MS/MS method was unaffected. UPLC–MS/MS provides a relatively simple, sensitive, and rapid means of argatroban monitoring. It has successfully been applied to assess plasma argatroban concentrations in hospitalized patients and may provide a more accurate determination of argatroban concentrations than an activity-based assay in certain clinical conditions.

1. Introduction

Heparin-induced thrombocytopenia (HIT) is a complication occurring in approximately 1–5% of patients treated with heparin [1,2], and requires the immediate replacement of heparin therapy with an alternative, rapidly active anticoagulant such as argatroban [3]. In contrast to heparins, which require formation of heparin–antithrombin–thrombin complexes to block thrombin activity [4], argatroban is a direct thrombin inhibitor (DTI) which reversibly binds to the thrombin active site. Derived from L-arginine, argatroban was licensed by the Food and Drug Administration for prophylaxis or treatment of thrombosis in patients with HIT and, in 2002, was approved for use during percutaneous coronary interventions in patients who have or are at risk for developing HIT.

Argatroban is administered intravenously, with steady-state blood levels and anticoagulant effect of argatroban usually obtained 1–3 h after initiation of therapy. Pharmacokinetic and pharmacodynamic studies have revealed that renal function, age, and sex do not have a clinical effect on metabolism, distribution, elimination, or anticoagulation of argatroban [5,6]. The main route of argatroban metabolism is hydroxylation and aromatization in the liver. This metabolism results in four known metabolites: M1, M2, M3, and M4 [7]. Of these metabolites, M1 is known to possess pharmacological activity, but is a significantly less potent thrombin inhibitor compared to argatroban [7].

Argatroban and other DTIs are frequently monitored using clotting-based methods such as the activated partial

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thromboplastin time (aPTT). However, monitoring of DTIs using aPTT can be problematic for several reasons. For example, the aPTT is susceptible to interference from lupus inhibitors [10], clotting factor inhibitors, and factor deficiencies which can all lead to overestimation of DTI anticoagulation. Patients being bridged to oral anticoagulants are also challenging to monitor with clotting-based assays because of the multiple anticoagulants on board. Argatroban-treated patients are typically transitioned to oral anticoagulants, such as warfarin, following recovery from their acute HIT [8]. Finally, other factors independent of DTI level, such as aPTT reagent selection and lot number can shorten or prolong the aPTT, resulting in an inaccurate estimation of these anticoagulants (reviewed in [9]). In recent years, commercially available indirect clotting-based methods have also been developed for the determination of DTIs [10].

Herein, we describe a method for direct quantitation of argatroban in plasma samples using a rapid ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) assay with a greater analytical measurement range than previously reported LC–MS/MS methods [6,7,11]. The UPLC–MS/MS method is compared to a commercially available, Hemoclot Thrombin Inhibitors [HTI] (HYPHEN BioMed) indirect activity-based clotting assay for argatroban measurement and recommendations are made with regards to clinical utility and advantages of each method.

2. Experimental

2.1. Chemicals and reagents

Argatroban was obtained from GlaxoSmith-Kline (Philadelphia, PA, USA) at a concentration of 100 mg/mL. Diclofenac, the primary internal standard (IS), and analytical or chromatography grade ammonium acetate and methanol were obtained from Sigma–Aldrich (St. Louis, MO, USA). Formic acid was obtained from Fisher Scientific (Fair Lawn, NJ, USA). De-ionized water was used throughout the study. Drug-free plasma was obtained from the Emory University Hospital blood bank, and was pooled from three separate sources. The Hemoclot Thrombin Inhibitors [HTI] assay was purchased from HYPHEN BioMed (distributed by Aniara Inc., Mason, OH, USA) and performed on a Dade Behring BCS instrument according to manufacturer's instructions. The indirect HTI clotting-based argatroban assay measures argatroban concentration based on inhibition of a constant and defined amount of highly purified human thrombin.

2.2. Preparation of calibration standards and stock solutions

Solutions were prepared as previously described [12] with the following modifications. The stock solutions of argatroban (primary standard, 100 mg/mL; secondary standard, 1 mg/mL; tertiary standard 100 μg/mL) and the IS diclofenac (10 mg/mL) were prepared in 50% deionized water/50% methanol and were stored at −80 °C and −20 °C, respectively. The standard working solutions of argatroban (100 μg/mL, 10 μg/mL, 1 μg/mL, and 0.1 μg/mL) and diclofenac (0.5 μg/mL) were prepared by dilution of stocks in methanol. Calibration standards were prepared by spiking drug free human plasma with the appropriate working solution of argatroban to yield concentrations of 0, 0.003, 0.04, 0.4, 0.7, 2.0, and 4.0 μg/mL. Quality control samples were prepared at concentrations of 0.009, 0.03, 0.3, and 3.0 μg/mL from different stock solutions than those used to prepare the calibrators.

2.3. Sample preparation

Blood was collected in 3.2% buffered sodium citrate tubes and plasma was separated by centrifugation at 4000 × *g* for 10 min. A 100 μL portion of patient plasma, calibrator, or control was added to 500 μL of methanol containing the IS at 0.2 μg/mL. The sample was vortexed for 2 min and then centrifuged at 11,356 × *g* for 10 min. 500 μL of the supernatant was transferred to a glass tube (15 mm × 75 mm), dried under nitrogen gas at 40 °C, and the residue was re-dissolved in 300 μL of mobile phase A (2 mM ammonium acetate in water; 0.1% formic acid). After brief vortexing, 150 μL aliquot was transferred to an auto-sampler vial and 4 μL injected into the UPLC–MS/MS system.

2.4. Calibration curve

Calibration standards were prepared and assayed as described above. Calibration curves were constructed by plotting the ratios of the peak area response for the argatroban and diclofenac peaks (i.e., argatroban AUC/diclofenac AUC) versus argatroban concentration. The calibration equation was obtained by $1/x$ weighted linear least squares regression analysis.

2.5. UPLC–MS/MS conditions

Separation was achieved using an ACQUITY UPLC BEH, 1.7 μm, 2.1 mm × 50 mm analytical column (Waters Corporation, Milford, MA, USA) heated to a constant 40 °C, with a constant flow rate of 250 μL/min and an instrument-controlled gradient, starting with 70% solvent A (0.1% formic acid, 2 mM ammonium acetate in water) and 30% solvent B (0.1% formic acid, 2 mM ammonium acetate in methanol). The initial solvent composition was held for 0.5 min, after which % B was gradually increased to 90% over 2.5 min, and then further increased to 100% B over 1.0 min. The solvent B composition was then recycled back to 30% over 0.5 min and held for a 60 s wash before the next injection, for a total run time of 5.0 min per injection/sample. Between injections, the sampling needle was washed for 30 s each with a weak wash (95% water, 5% methanol, 0.1% formic acid) and strong wash (95% methanol, 5% water, 0.1% formic acid).

The ACQUITY UPLC was coupled to a TQD mass spectrometer (Waters, Corporation Milford, MA, USA) operated in positive electrospray ionization mode. Main working parameters were set as follows: cone 50.00 V, collision energy 20.0 keV for argatroban and 40.0 keV for diclofenac, and desolvation temperature 150 °C. Two ion transitions were monitored per compound in multiple reaction monitoring (MRM) mode (argatroban: 509.4 > 384.4 (quantifier); 509.4 > 237.3 (qualifier) and diclofenac: 296.1 > 250.5 (quantifier); 296.1 > 214.2 (qualifier)). Multiple transitions are commonly monitored in LC–MS/MS assays, with the most abundant ion transition used for quantitation, and the second transition used as a qualifier. Data was processed and calibration curves were generated using TargetLynx Software (Waters Corporation, Milford, MA, USA).

2.6. Validation of the method

2.6.1. Matrix effect and recovery

Plasma matrix effects were evaluated by a method adapted from Matuszewski et al. [17] in which the peak areas obtained for 0.1 μg/mL of argatroban spiked in pre-extracted pooled plasma were compared to those generated with the same concentration of argatroban spiked in the aqueous mobile phase A at the same concentration. Percent ion suppression was calculated as: $(A_M - A_p)/A_M \times 100$, where A_M is the peak area of the argatroban spiked in mobile phase A and A_p is the mean peak area of the argatroban spiked in plasma. Recovery was evaluated at three concentrations (0.009, 0.1, and 1.5 μg/mL) by spiking drug free plasma with argatroban either before or immediately after the extraction procedure. Percent recovery was calculated as: $A_{pre}/A_{post} \times 100$,

where A_{pre} is the average peak area of samples spiked with argatroban before extraction, and A_{post} is the average peak area of samples spiked with argatroban after extraction ($n = 5$).

2.6.2. Sensitivity and linearity

The lower limit of quantitation (LLOQ) was defined as the lowest argatroban analyte concentration which upon repeated sampling ($n = 5$), generated a coefficient of variation (CV) ≤ 15%, and a S/N ratio > 10. Linearity was confirmed over the calibration range.

2.6.3. Imprecision and carryover

Intra-assay imprecision was evaluated by repeated within-run measurement ($n = 20$) of the 0.009, 0.03, 0.30, and 3.0 μg/mL quality controls. The same controls were also measured 20 times over 4 days to assess inter-assay variability. Carryover was evaluated by averaging results for the 0.03 μg/mL standard assayed twice before (C_i) and twice after (C_f) running the 3.0 μg/mL standard. Percentage carryover was determined as $[(C_f - C_i)/C_i] \times 100$.

2.6.4. Interference

Hemolysis interference was investigated by assaying hemolyzed plasma samples spiked with 1.0 μg/mL of argatroban in comparison with unhemolyzed plasma spiked with the same amount of drug. Hemolyzed plasma was generated by freezing one of two drug free whole blood samples obtained from a single healthy donor at −20 °C prior to centrifugation. Various levels of hemolysis were obtained by serial dilution of the resulting hemolyzed and the non-hemolyzed plasma samples. The concentration of hemoglobin in plasma samples was determined on a COULTER® LH 750 Hematology Analyzer (Beckman Coulter, Inc., CA, USA).

2.7. Method comparison

Pooled normal plasma, Factor XIII deficient plasma (George King Bio-Medical, Inc., Overland Park, KS, USA), and residual patient plasma samples found to contain high levels of fibrinogen (obtained from the Emory Clinical Translational Research Laboratory Biobank and approved by the Emory Institutional Review Board (IRB00018010)) were spiked in triplicate with argatroban at final concentrations of 0.3, 0.8, and 1.50 μg/mL and analyzed by UPLC–MS/MS and HTI on the Dade Behring BCS.

Residual plasma samples from hospitalized patients ($n = 10$) treated with argatroban were obtained from the Emory Clinical Translational Research Laboratory Biobank and analyzed by the UPLC–MS/MS and HTI methods.

3. Results and discussion

3.1. Method validation

Chromatograms of plasma spiked with argatroban and internal standard, diclofenac, are shown in Fig. 1A and B, respectively. Argatroban and diclofenac eluted at approximately 1.88 and 2.06 min, respectively. Representative mass spectra showing the selected precursor and product ions used for detection of argatroban and diclofenac are included as insets in Fig. 1A and B, respectively. The precursor ion of argatroban displays an m/z of 509.4, and its primary and secondary product ions an m/z of 384.4 and 237.3, respectively (Fig. 1A, inset). The precursor ion of diclofenac shows an m/z of 296.1, and its primary and secondary product ions an m/z of 250.5 and 214.2, respectively (Fig. 1B, inset).

3.1.1. Linearity and LLOQ

The assay was linear over the concentration range of 0.003–4.0 μg/mL. The mean ($n = 5$) correlation coefficient from five separate standard curves was 0.99 ($\pm 2.6 \times 10^{-6}$) with an average slope of 0.99 ($\pm 3.9 \times 10^{-5}$) and intercept of 0.0025 (±0.0013). The LLOQ was 0.003 μg/mL with an imprecision of 10.4%, and S/N ratio of 42, which is more sensitive than previously reported HPLC–MS/MS methods [6,7].

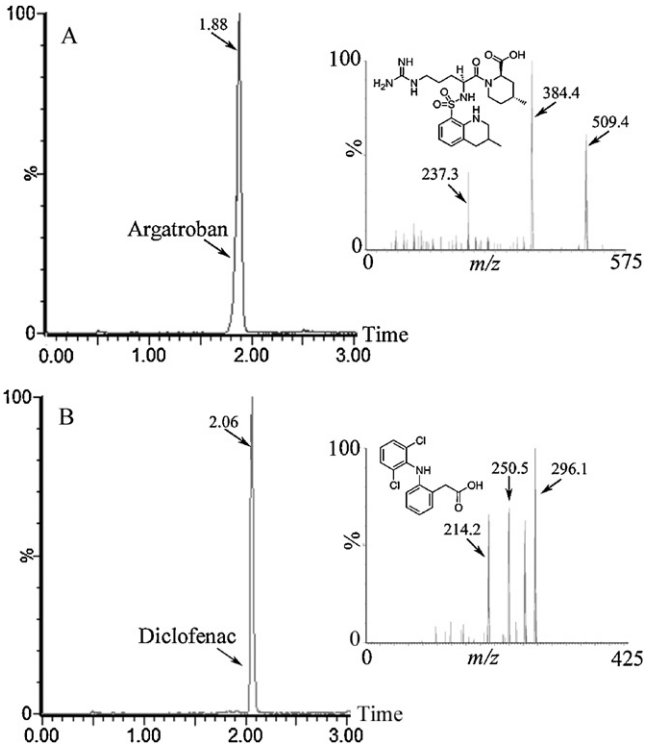

Fig. 1. UPLC–MS/MS chromatograms of plasma spiked with argatroban (A) and diclofenac (B). Full scan mass spectra and chemical structures of argatroban (A, inset) and diclofenac (B, inset).

3.1.2. Imprecision and carryover

Intra- and inter-assay imprecision data of plasma QC samples are summarized in Table 1. Intra-assay imprecision ranged from 3.4 to 9.9%, while inter-assay imprecision ranged from 8.4 to 11.7%. Percent recoveries ranged from 85.4% at 0.009 μg/mL to 95% at 1.5 μg/mL.

Carryover studies showed minimal carryover (≤1.5%) following injection of samples with concentrations as high as 3.0 μg/mL of argatroban.

3.1.3. Ion suppression and interference

No significant ion suppression (>5%) was observed for argatroban spiked into drug free plasma compared to argatroban spiked into aqueous mobile phase. Hemolyzed plasma can be visualized beginning at ~1% hemolysis (0.3 g/dL) [13]. In our assay, hemolyzed plasma with measured hemoglobin concentrations of up to 0.4 g/dL did not significantly interfere with argatroban quantification (<10%) (Table 2). However, hemoglobin concentrations greater than or equal to 0.7 g/dL, corresponding to 3% hemolysis or

Table 1
UPLC–MS/MS method intra- and inter-assay imprecision.

Concentration (μg/mL)	Imprecision (CV%)	
	Intra-assay ($n = 20$)	Inter-assay ($n = 20$)
0.009	3.4	7.3
0.03	9.9	8.4
0.3	6.7	11.7
3.0	6.9	10.5

Table 2
Effects of increasing hemoglobin (Hgb) concentrations on UPLC–MS/MS measurement of plasma spiked with 1 μg/mL argatroban.

Hgb concentration (g/dL)	Argatroban concentration (μg/mL)	Difference (%)
0.0	1.04	0
0.4	0.98	−6
0.7	0.8	−23
1.3	0.78	−25
2.1	0.79	−24

more, significantly decreased the measured argatroban concentration by up to 25%.

3.2. Method comparison

The UPLC–MS/MS method was compared to a commercially available HTI activity-based method for argatroban quantification using plasma samples from 10 different hospitalized patients administered argatroban. The measurements for two of these samples were above the analytical measurement range (AMR) of the HTI assay (>2.0 μg/mL). The extended range of our assay allowed us to calculate the concentration of argatroban in these two samples at 1.8 and 3.9 μg/mL. The correlation between UPLC–MS/MS and HTI measured argatroban concentrations using the remaining 8 plasma samples is shown in Fig. 2A. UPLC–MS/MS argatroban concentrations correlated well with the HTI method (Fig. 2A), yielding a slope of 1.0, an intercept of −0.15, and r^2 of 0.98. The bias between the two methods is reflected in a Bland–Altman plot (Fig. 2B). A limitation of our analysis is the small number of patient samples tested. The single sample at a high concentration may have a disproportionate influence on the slope and intercept of the regression equation. Testing additional samples collected from patients dosed with argatroban will be important to draw a more statistically significant conclusion about HTI and UPLC–MS/MS method agreement.

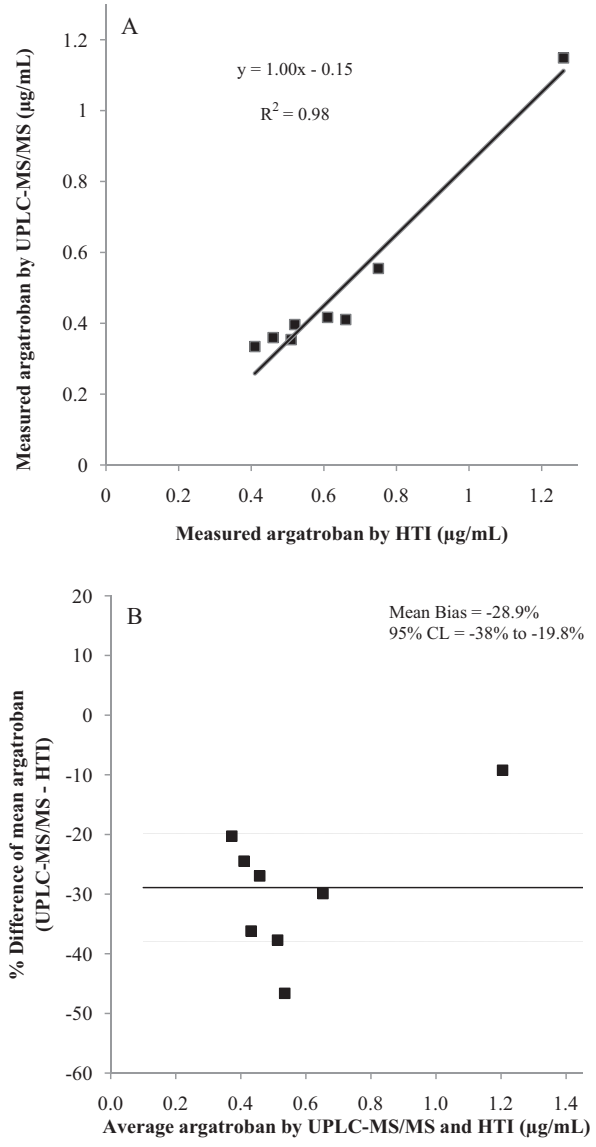

Fig. 2. (A) Comparison of UPLC–MS/MS versus HTI for argatroban quantification using 8 plasma samples from patients treated with argatroban. The regression equation was constructed using Deming regression analysis. (B) Bland–Altman bias plot showing the percentage difference between the measured UPLC–MS/MS and HTI concentrations versus the mean of the two methods.

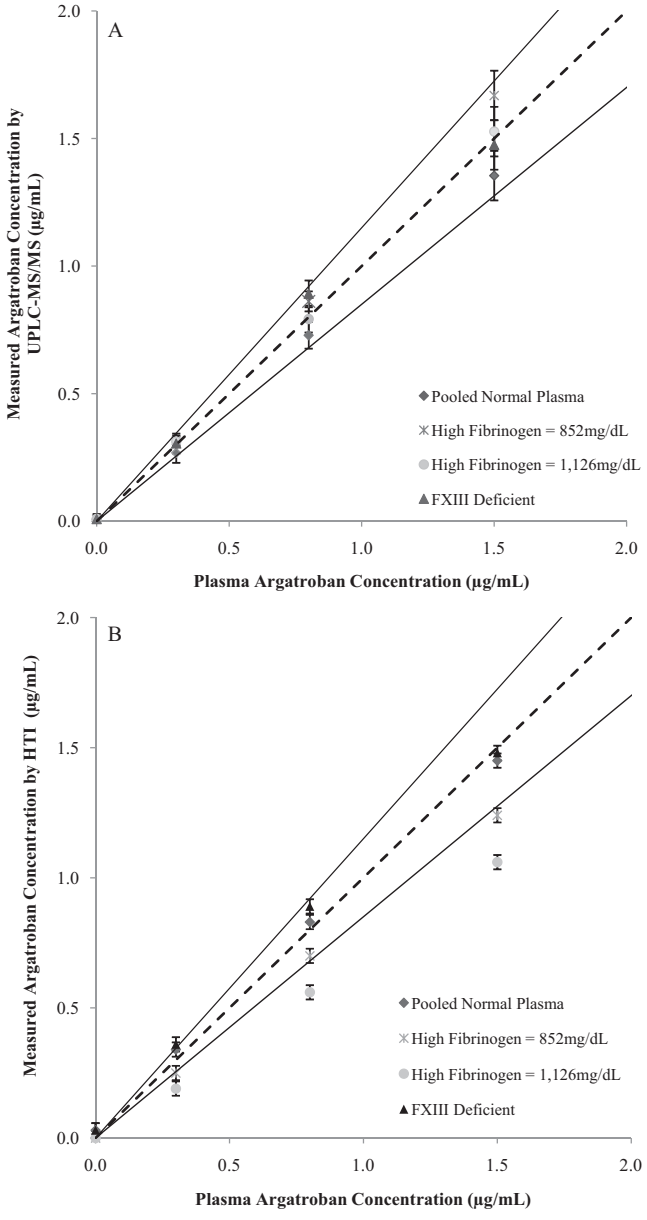

Fig. 3. Measured argatroban concentrations obtained by (A) UPLC–MS/MS and (B) HTI for plasma samples spiked with increasing amounts of argatroban. Error bars represent one standard deviation. The solid lines indicate ±15% from the line of identity which is indicated by a dashed line.

We hypothesized that the presence of one or more pharmacologically active metabolites, such as the M1 metabolite, not detected by UPLC–MS/MS, could potentially account for some of the differences seen in argatroban quantitation between the HTI and UPLC–MS/MS analysis (Fig. 2). However, the M1 metabolite was not detected in full scan spectra of argatroban treated patient samples (data not shown). Effects of patient sample matrix and tube type (sodium citrate, lithium-heparin, and EDTA) were also investigated, but did not contribute to the negative bias observed (data not shown).

Unlike UPLC–MS/MS, which provides a direct measurement of drug concentration, indirect activity-based quantification methods are prone to interference from a variety of factors and conditions, potentially leading to falsely prolonged or shortened clotting times, and miscalculation of DTI concentrations. Such conditions include abnormal levels of fibrinogen [14]. Fig. 3A demonstrates that the UPLC–MS/MS measurements are within ±15% of the line of identity for the spiked pooled normal plasma, two plasma containing high concentrations of fibrinogen, and Factor XIII deficient plasma. These conditions do not affect argatroban determination by UPLC–MS/MS. In contrast, the HTI method significantly underestimates argatroban concentrations in the presence of high fibrinogen at concentrations of 852 mg/dL and 1126 mg/dL (Fig. 3B). Such underestimation is similar to the effect previously described by Love et al. [15] in which a 10% decrease in thrombin time was reported for argatroban-spiked plasma samples containing >600 mg/dL fibrinogen. Because there are no reversal agents for DTIs, elevated levels of these drugs carry the risk of life-threatening bleeding complications [16]. Thus, underestimation of argatroban may exacerbate certain clinical situations and lead to extended coagulopathy [18]. The direct UPLC–MS/MS assay was unaffected by elevated fibrinogen levels and thus, should provide a more accurate determination of argatroban concentrations in patients with abnormally high fibrinogen levels (Fig. 3A). The activity-based HTI method and the UPLC–MS/MS method were unaffected by deficiency of Factor XIII (Fig. 3A).

4. Conclusion

The UPLC–MS/MS method offers a potential alternative to the clotting-based HTI assay. Argatroban concentrations, as quantified by the UPLC–MS/MS method, correlated well with argatroban levels measured by the HTI clotting method in the patient plasma samples from patients treated with argatroban. Extending the AMR using UPLC–MS/MS (0.003–4.0 μg/mL versus 0–2.0 μg/ml for HTI) offers the advantage of reduced analytical time, as patient samples with >2.0 μg/mL of argatroban (but <4.0 μg/mL) do not have to be diluted and reanalyzed. The UPLC–MS/MS method was unaffected by factors such as high fibrinogen which can lead to inaccurate determinations of argatroban concentrations by indirect clotting-based methods. Direct measurement of argatroban using a direct approach may provide a more accurate determination of argatroban levels in certain clinical situations known to increase fibrinogen levels, such as in diabetics, post menopausal women, in persons with a high body mass index, and in smokers [18].

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