Simple LC-MS/MS method using core-shell ODS microparticles for the simultaneous quantitation of edoxaban and its major metabolites in human plasma

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Simple LC-MS/MS method using core-shell ODS microparticles for the simultaneous quantitation of edoxaban and its major metabolites in human plasma

Saki Ariizumi [a], Takafumi Naito [a,*], Kohei Hoshikawa [a], Shunta Akutsu [a], Masao Saotome [b], Yuichiro Maekawa [b], Junichi Kawakami [a]

[a] *Department of Hospital Pharmacy, Hamamatsu University School of Medicine,* [b] *Internal Medicine III, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan*

**Corresponding author: Takafumi Naito, Department of Hospital Pharmacy, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan*

Phone +81 53 435 2623, Fax +81 53 435 2764

e-mail: naitou@hama-med.ac.jp

Abstract

Edoxaban is mainly enzymatically converted to a 4-carboxylic acid form (4CA-EDX) and an *N*-desmethyl form (ND-EDX) in humans. This study aimed to establish a simple liquid chromatography-tandem mass spectrometry method using core-shell octadecyl silica (ODS) microparticles for the simultaneous quantitation of edoxaban and its two major metabolites in human plasma. Analytes extracted from plasma specimens by a one-step deproteinization were separated using a 2.6-μm core-shell ODS microparticulate column and linear acetonitrile-ammonium acetate gradient elution at a flow rate of 0.25 mL/min with a run time of 7 minutes. The mass spectrometer was operated in the positive ion multiple reaction monitoring mode. Plasma samples collected from 20 patients with atrial fibrillation were analyzed by the present method. The chromatograms of drug-free human plasma had no interfering peaks. The calibration curves of edoxaban, 4CA-EDX, and ND-EDX were linear over the concentration ranges of 1.25–160, 0.47–60, and 0.12–15 ng/mL, respectively. Their pretreatment recoveries and matrix factors were 88.7–109.0% and 87.0–101.6%, respectively. The intra- and inter-day accuracy and imprecision values were 85.9–112.8% and within 13.3%, respectively. The plasma concentrations of edoxaban, 4CA-EDX, and ND-EDX in the patients had ranges of 17.8–102, 1.67–25.7, and 0.685–5.34 ng/mL, respectively. All the analytes were measurable within their calibration curves. In conclusion, this validated method for the simultaneous determination of edoxaban and its major metabolites was successfully applied to plasma specimens obtained from patients with atrial fibrillation.

Key words:

Edoxaban; metabolites; LC-MS/MS; human plasma; pharmacokinetics

1. Introduction

Edoxaban, a direct oral anticoagulant that directly inhibits coagulation factor Xa (FXa), is commonly used for the prevention of ischemic stroke and systemic embolism and for the prevention and treatment of venous thromboembolism [1,2]. Although the balance between bleeding and thrombus formation needs to be maintained in edoxaban therapy, no global monitoring index of its anticoagulant effect is employed in clinical practices. A large variation of anti-FXa activity in patients treated with edoxaban for atrial fibrillation was recently reported [2–4].

The plasma concentration of edoxaban has a large interindividual variation [3]. Edoxaban shows moderate intestinal absorption with an oral bioavailability of approximately 60% and possesses an elimination half-life of around 10 hours [5,6]. Edoxaban is primarily converted to the 4-carboxylic acid form (4CA-EDX) by carboxylesterase-1 and to the *N*-desmethyl form (ND-EDX) by cytochrome P450 (CYP) 3A4/5 [7]. The anti-FXa activities of 4CA-EDX and ND-EDX are comparable with that of edoxaban [7], while little data on the pharmacokinetic profiles of the metabolites are available. The plasma concentration of edoxaban, which shows a positive correlation with the anti-FXa activity, cannot fully explain the variations of the anticoagulant effect in patients with atrial fibrillation [3]. The plasma exposures of the active metabolites in addition to edoxaban may be responsible for the interindividual variations in edoxaban efficacy and safety.

Plasma concentrations of edoxaban have been determined by various approaches [8–

12]. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to obtain high sensitivity in most measurement studies of edoxaban. The plasma concentrations of 4CA-EDX and ND-EDX are lower than that of edoxaban in humans [7,13]. Although LC-MS/MS is needed to measure the active metabolites in human plasma from the viewpoint of sensitivity and specificity, few validated methods are available for the simultaneous quantitation of plasma edoxaban and its metabolites in patients with atrial fibrillation. Recent analytical reports determined 4CA-EDX in addition to edoxaban in human plasma [14,15]. The acid dissociation constants of ND-EDX, edoxaban, and 4CA-EDX are presumed to decrease in this order, based on their chemical structures [16]. A long-term LC separation is generally required for the simultaneous quantitation of analytes with similar molecular weights but different polarities. An LC column with core-shell octadecyl silica (ODS) microparticles shows higher performance in separation and elution than that with fully porous particles [17]. The LC column potentially achieves the high sensitivity by sharpening chromatographic peaks and the short run time by decreasing elution volume due to decreasing the diffusion of each analyte into core-shell ODS microparticles.

This study developed a simple LC-MS/MS method using core-shell ODS microparticles for the simultaneous quantitation of edoxaban and its major metabolites in human plasma. In addition, we applied the method to pharmacokinetic analyses of edoxaban in patients with atrial fibrillation.

2. Material and methods

2.1. Chemicals

Edoxaban tosilate hydrate was purchased from Selleck Chemicals (Houston, TX, USA). 4CA-EDX and 6-deuterium-labeled edoxaban as an internal standard (IS) were sourced from Alsachim (Illkirch-Graffenstaden, France). ND-EDX hydrochloride was obtained from Molcan Corporation (Toronto, Canada). HPLC-grade acetonitrile and methanol, ammonium acetate, and triethylamine were provided by Fujifilm Wako Pure Chemicals (Osaka, Japan).

2.2. Solutions

Methanol solutions of edoxaban (50 μg/mL), 4CA-EDX (20 μg/mL), ND-EDX (50 μg/mL), and IS (20 μg/mL) were stocked at 4°C. The plasma concentrations of calibration standards were 1.25–160, 0.47–60, and 0.12–15 ng/mL for edoxaban, 4CA-EDX, and ND-EDX, respectively. Lower limit of quantification (LLOQ) and three quality control (QC) samples were prepared as plasma spiked to concentrations of 1.25, 1.6, 32, and 128 ng/mL for edoxaban, 0.47, 0.60, 12, and 48 ng/mL for 4CA-EDX, and 0.12, 0.15, 3.0, and 12 ng/mL for ND-EDX.

2.3. Sample preparation

Blood specimens were collected into tubes containing ethylenediaminetetraacetic acid (EDTA) dipotassium salt. One-hundred microliters of IS solution (10 ng/mL in acetonitrile) and 300 μL of acetonitrile were added to 100 μL of EDTA-treated plasma. The mixture was centrifuged at

10,000 × *g* for 20 minutes at 4°C and then 400 μL of the supernatant was dried with a vacuum evaporator. The residue was reconstituted with 200 μL of mobile phase and filtrated before injection into the LC system.

2.4. Chromatographic conditions

The pretreated samples were injected into a Nexera X2 system (Shimadzu Corporation, Kyoto, Japan). Edoxaban and its metabolites were chromatographically separated at 40°C using a 2.6-μm core-shell ODS microparticulate column (Kinetex C18, 100 mm length × 2.1 mm inner diameter, Phenomenex, Torrance, CA, USA) with a guard column (SecurityGuard Ultra Cartridge, Phenomenex). The initial mobile phase was 11.5% acetonitrile containing 5 mM ammonium acetate in water. The ratio of acetonitrile was linearly increased to 35% in 0.5 minutes, held at 35% for 5.9 minutes, and then linearly returned to 11.5% in 0.3 minutes at a constant flow rate of 0.25 mL/min with a total run time of 7 minutes. The autoinjector temperature and the injection volume were set at 4°C and 6 μL, respectively.

2.5. Mass spectrometer conditions

The column effluent was loaded into a triple quadrupole mass spectrometer (LCMS-8050, LabSolutions LCMS ver. 5.91, Shimadzu Corporation) with an electrospray probe from 2.5 to 7 minutes. The ion transitions were scanned in the positive ion multiple reaction monitoring mode with a dwell time of 100 milliseconds for each analyte: edoxaban, *m/z* = 548.10/366.25;

4CA-EDX, 520.90/339.15; ND-EDX, 533.80/352.25; and IS, 554.10/372.25. The turbo ion-spray ionization was carried out at a voltage of 4.0 kV and temperature of 300°C. The pressure of the collision-induced dissociation gas was 4 kPa; the flow rates of the drying gas, nebulizer gas, and heating gas were set at 10 mL/min, 3 mL/min, and 10 mL/min, respectively. Collision energies, entrance potential, and collision cell exit potential were set at -30, -21, and -18 volts for edoxaban, -26, -21, and -16 volts for 4CA-EDX, -26, -21, and -17 volts for ND-EDX, and -30, -21, and -18 volts for IS.

2.6.1. Analytical performance

Selectivity of the method was determined by measuring six independent plasma specimens obtained from atrial fibrillation patients treated without edoxaban. Calibration curves for the plasma concentrations of edoxaban and its metabolites were calculated using least squares linear regression with a weighting factor of x^{-2}. The LLOQ samples (n = 6) were measured to test whether their coefficients of accuracy (% error) and imprecision (% relative standard deviation, RSD) were within 20%. The dilution effects were assessed by comparing the peak area ratios of plasma samples spiked to the upper-limit concentration of each calibration curve with those prepared by 5-fold dilution (n = 3). The pretreatment recovery was evaluated as the peak area ratio of analytes extracted from spiked plasma to those spiked after plasma extraction. The matrix effect on ionization at each batch was defined as the peak area ratio of analytes spiked after plasma extraction to those prepared in mobile phase. The pretreatment recovery

and the matrix effect were assessed using three QC plasma samples (n = 3). The intra- and inter-day accuracy and imprecision were determined by analyzing six sets of respective QC samples. Carry-over was evaluated by analyzing a drug-free plasma sample after measurement of a plasma sample at the highest concentration of each calibration range (n = 3).

2.6.2. Stability of analytes

The stability of analytes in plasma was determined by comparing peak areas after a 6-hour incubation at 4°C or at room temperature with their initial values. The long-term stabilities were defined as the changes in peak areas of plasma samples stored at -80°C for one month. The effects of the freeze and thaw processes in plasma were measured after two freeze-thaw cycles. The analytical stabilities of analytes were assessed by comparison between peak areas after a 24-hour storage in mobile phase at 4°C and their initial values. Analytes in methanol solution were remeasured after three months for evaluation of the stock stability. The respective stabilities were assessed for three QC samples (n = 3).

2.7. Clinical application

The study protocol was approved by the Ethics Committee of Hamamatsu University School of Medicine (18-134). A total of 20 Japanese inpatients treated with oral edoxaban tosilate hydrate tablets (Lixiana, Daiichi Sankyo Co. Ltd., Tokyo, Japan) for atrial fibrillation at Hamamatsu University Hospital were enrolled. The patients received 15, 30, or 60 mg of

edoxaban once daily. Blood specimens from a forearm vein were collected into tubes containing EDTA dipotassium salt just before dosing on day 3 or later. Variations in plasma exposures of edoxaban and its metabolites were assessed as dose and body weight-corrected plasma concentration (ng/mL per mg/kg). Metabolic ratios were defined as the plasma concentration ratios of the metabolites to edoxaban.

3. Results

3.1. Separation and Selectivity

Figure 2 shows LC-MS/MS chromatograms of drug-free plasma, edoxaban-free patient plasma, drug-free plasma spiked with analytes, and a plasma specimen of an atrial fibrillation patient treated with oral edoxaban. Edoxaban, 4CA-EDX, ND-EDX, and IS were eluted at 5.2, 3.4, 4.1, and 5.1 minutes, respectively, with a total run time of 7 minutes. No peaks interfering with analytes were observed in six independent edoxaban-free plasma specimens from atrial fibrillation patients.

3.2. Calibration curves, sensitivity, and dilution effects

Calibration curves were linear over the plasma concentration ranges of 1.25–160, 0.47–60, and 0.12–15 ng/mL for edoxaban, 4CA-EDX, and ND-EDX, respectively. Their correlation coefficients were more than 0.999. The LLOQs of edoxaban, 4CA-EDX, and ND-EDX in human plasma were 1.25, 0.47, and 0.12 ng/mL, respectively. The intra-day accuracies and

imprecisions for LLOQ samples were 98.7% and 3.7% for edoxaban, 105.2% and 6.8% for 4CA-EDX, and 95.1% and 9.8% for ND-EDX. The inter-day values were 112.7% and 12.7% for edoxaban, 107.7% and 8.9% for 4CA-EDX, and 100.8% and 12.3% for ND-EDX. The peak area ratios with respect to the dilution effects were within 88.0–110.3% for all the analytes.

3.3. Pretreatment Recovery, Matrix effect, and Carry-over

The pretreatment recoveries in plasma were 96.6–107.1%, 88.7–103.6%, 103.8–109.0%, and 100.7–104.6% for edoxaban, 4CA-EDX, ND-EDX, and IS, respectively. No matrix effects were observed in plasma; the matrix factors had ranges of 98.4–99.2%, 87.0–95.7%, 87.0–91.7%, and 97.2–101.6% for edoxaban, 4CA-EDX, ND-EDX, and IS, respectively. No quantifiable peak was detected in the carry-over tests.

3.4. Assay accuracy and imprecision

Table 1 summarizes the intra- and inter-day accuracy and imprecision in human plasma. The intra- and inter-day accuracies of edoxaban, 4CA-EDX, and ND-EDX were 89.4–109.2% and 89.6–104.8%, 85.9–112.8% and 86.5–104.2%, and 91.2–102.7% and 95.1–99.9%, respectively. Their corresponding imprecisions were 2.1–6.3% and 2.7–11.8%, 2.0–8.4% and 3.4–10.1%, and 8.6–13.3% and 8.3–8.8%, respectively.

3.5. Stability

Edoxaban and its metabolites in plasma specimens were stable at room temperature (% of initial value, 97.2–107.9% for edoxaban, 94.7–106.1% for 4CA-EDX, and 87.3–94.3% for ND-EDX) and at 4°C (% of initial value, 99.0–105.8% for edoxaban, 98.7–106.1% for 4CA-EDX, and 87.9–104.2% for ND-EDX) for up to 6 hours, respectively. They were also stable in plasma at -80°C for up to 1 month (% of initial value, 88.9–104.3% for edoxaban, 90.4–98.9% for 4CA-EDX, and 93.7–113.0% for ND-EDX). No effect was observed after two freeze-thaw cycles (% of initial value, 92.5–101.7% for edoxaban, 93.4–101.9% for 4CA-EDX, and 85.6–101.2% for ND-EDX). Edoxaban and its metabolites in mobile phase were stable at 4°C for up to 24 hours (% of initial value, 96.3–98.6% for edoxaban, 101.6–105.4% for 4CA-EDX, and 103.4–113.8% for ND-EDX). The chemicals stored in methanol were stable at 4°C for up to 3 months (% of initial value, 97.9% for edoxaban, 101.1% for 4CA-EDX, and 96.3% for ND-EDX).

3.6. Application to pharmacokinetic study

Figure 3 shows the plasma concentrations of edoxaban and its metabolites in 20 atrial fibrillation patients. The plasma concentrations of edoxaban, 4CA-EDX, and ND-EDX had ranges of 17.8–102, 1.67–25.7, and 0.685–5.34 ng/mL, respectively. They were all measurable within each calibration range. Large interindividual variations were observed in the dose and body weight-corrected plasma concentrations of edoxaban, 4CA-EDX, and ND-EDX, which had the median and interquartile range (IQR) of 109 and 65.0–122, 15.7 and 8.31–26.3, and 4.05 and 1.72–4.88 ng/mL per mg/kg, respectively. The atrial fibrillation patients also showed

large variations in the metabolic ratios of 4CA-EDX and ND-EDX to edoxaban, which were 0.175 and 0.125–0.234, and 0.0436 and 0.0321–0.0495, respectively, as the median and IQR.

4. Discussion

To the best of our knowledge, this is the first report on a simultaneous quantitative method of edoxaban and its two major metabolites in human plasma using an LC-MS/MS and its application in patients with atrial fibrillation. The analytes were extracted from plasma specimens through a simple pretreatment method with one-step deproteinization. Complete separation was achieved by a linear gradient program using a 2.6-μm core-shell ODS column with a run time of 7 minutes. The calibration curves of edoxaban and its metabolites in plasma were linear. The analytical performance of the method met the requirements of the U.S. FDA guidance [18]. The plasma concentrations of the analytes in 20 patients with atrial fibrillation were all measurable within each calibration range. These data confirmed that the method was suitable for clinical situations.

This study employed deproteinization with a small amount of acetonitrile for the pretreatment of plasma samples. The present method was highly acceptable in terms of pretreatment recovery and matrix effect. Several earlier LC-MS/MS methods for the determination of edoxaban in human plasma involved solid-phase extraction or organic extraction with diethyl ether under alkaline conditions for sample pretreatment [8–11,14]. In the current study, a simple acetonitrile-precipitation without pH adjustment was used in the

plasma pretreatment for the simultaneous quantification of edoxaban and its major metabolites. Our pretreatment procedure can be beneficial for clinical application in terms of cost, time, simplicity, and less environmental loading in comparison with these earlier methods.

The LC column effluent was introduced into the MS/MS system for 4.5 minutes between 2.5–7 minutes in a total run time of 7 minutes. No peak that interfered with analytes was observed in the chromatograms. Edoxaban has an acid dissociation constant of 6.7, which is presumed to lie between the values of 4CA-EDX which has a carboxylate structure and ND-EDX which has a tertiary amine structure [16]. The column retention of compounds with different polarities varies according to the aqueous-organic solvent ratio of the mobile phase. In the present method, both adequate retention intervals and short elution times were obtained using a core-shell ODS column with a simple and elaborate gradient program. A thin diffusion layer formed in the column is able to increase the separation efficacy of analytes with different polarities and shorten their elution times [17]. Although Bathala et al. [13] identified edoxaban, 4CA-EDX, and ND-EDX in plasma using an LC-MS/MS, this method with long-term separation cannot apply to clinical laboratory practice. Our method which can be simultaneous and short-term quantitation of edoxaban and its two major active metabolites would be needed for clinical use.

The LLOQs of edoxaban, 4CA-EDX, and ND-EDX in human plasma were 1.25, 0.47, and 0.12 ng/mL, respectively. The LLOQ of edoxaban in the present method was similar to that in earlier methods without quantitative conditions of the metabolites [8–10,12]. The plasma

concentration of edoxaban is presumed to be around 3 ng/mL in some special patients treated with 15 mg of edoxaban once daily [19]. The sensitivity for plasma edoxaban in our method is suitable for application to clinical laboratory practice. A high-sensitivity method was needed to determine the plasma exposures of 4CA-EDX and ND-EDX, which were lower than that of edoxaban itself [7,13]. Edoxaban and its metabolites in the LLOQ samples were successfully quantified with our optimized method as their accuracy and imprecision were 95.1–112.7% and 6.8–12.7%, respectively. Calibration curves for the determination of edoxaban and its metabolites were linear over a sufficiently wide range for their trough plasma concentrations.

The intra- and inter-day accuracy and imprecision for edoxaban and its metabolites in human plasma were 85.9–112.8% and 2.0–13.3%, respectively. This method demonstrated excellent reproducibility and reliability. Gosselin *et al*. reported the instability of edoxaban in plasma after 24-hour incubation at room temperature [20]. In our study, edoxaban and its metabolites were fairly stable in human plasma at room temperature and at 4°C for up to 6 hours, which seems to be long enough for sample preparation. More than a hundred samples can be assayed together in the same batch, since the analytes were stable for up to 24 hours in the mobile phase at 4°C. The method possesses a clear advantage with respect to its clinical applicability in terms of the stability for continuous measurement of edoxaban and its metabolites.

The plasma concentrations of edoxaban, 4CA-EDX, and ND-EDX were 17.8–102, 1.67–25.7, and 0.685–5.34 ng/mL, respectively, in 20 atrial fibrillation patients. They were all

measurable within each calibration curve. Suzuki *et al*. showed that the trough plasma exposure of edoxaban was 2.3–70.6 ng/mL in atrial fibrillation patients treated with 30–60 mg of oral edoxaban [19]. The plasma concentration range of edoxaban in our study population was slightly higher than that in an earlier report, probably due to the relatively poor renal function of the present patients. The trough plasma concentration of edoxaban was reported to reach approximately 150 ng/mL in a patient treated with oral edoxaban 60 mg once daily [3]. The 5-fold dilution procedure in our method was acceptable for the determination of relatively high plasma concentrations of edoxaban. The present analytical method is potentially suitable to a broad patient population with atrial fibrillation.

Although the dose of edoxaban chosen was based on the body weight, renal function, and concomitant drug use of the patients, the plasma concentrations of edoxaban, 4CA-EDX, and ND-EDX exhibited large variations in the patients with atrial fibrillation. The dose and body weight-corrected plasma concentrations of edoxaban and its metabolites and their metabolic ratios also had large interindividual variations. Edoxaban is predominantly metabolized by carboxylesterase-1 and CYP3A4/5 [7]. In addition, edoxaban is a substrate of the efflux transporter P-glycoprotein, while 4CA-EDX is recognized by the hepatic uptake transporter organic anion transporter protein 1B1 [21]. The variability in plasma edoxaban exposure is potentially attributed to the difference in activities of metabolic enzymes and transporters related to the absorption and excretion of edoxaban and its metabolites between patients.

The current study has a few limitations. First, the applicability of the present method was confirmed for plasma samples obtained from only patients with atrial fibrillation. Edoxaban is also used for the prevention and treatment of vein thrombosis and pulmonary embolism at a dose of 15–60 mg once daily. Thus, patients with venous thromboembolism are assumed to exhibit plasma edoxaban concentrations that are close to those with atrial fibrillation. The usefulness of our method for venous thromboembolism patients needs to be further investigated. Second, the stability of edoxaban and its metabolites in human plasma satisfied the U.S. FDA guidance at room temperature and 4°C for up to 6 hours. Although edoxaban has a chemically reactive amide bond, a longer stability in plasma potentially makes the method more useful in clinical practice. In this study, blood samples needed to be immediately centrifuged and stored at -80°C before a simple and rapid pretreatment procedure. Third, the current study did not assess whether the method could be applied to special populations with impaired hepatic function or renal function. The plasma concentrations of the analytes were all measurable within each calibration range in the enrolled patients with IQR of 0.80–1.15 mg/dL for serum creatinine and 0.7–1.0 mg/dL for total bilirubin. The upper-limit plasma concentration of edoxaban was 128 ng/mL in the calibration range without the 5-fold dilution procedure. This method with wide measurement will be applied to the determination of plasma edoxaban and its metabolites in patients with low metabolism and excretion.

5. Conclusions

This study developed a simple LC-MS/MS method using core-shell ODS microparticles for the simultaneous quantitation of edoxaban and its major metabolites in human plasma. In addition, we applied the method to pharmacokinetic analyses of edoxaban in patients with atrial fibrillation.

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Figure legends

Figure 1. Mass spectra and mass-to-charge (*m/z*) of (A) edoxaban, (B) 4CA-EDX, and (C) ND-EDX.

Edoxaban, *m/z* 548.10/366.25; 4CA-EDX, 520.90/339.15; and ND-EDX, 533.80/352.25.

Figure 2. LC-MS/MS chromatograms of edoxaban and its metabolites.

(A) Human drug-free plasma, (B) a plasma specimen obtained from an atrial fibrillation patient treated without edoxaban, (C) human drug-free plasma spiked with 10 ng/mL edoxaban, 3.75 ng/mL 4CA-EDX, and 0.94 ng/mL ND-EDX, and (D) a plasma specimen obtained from an atrial fibrillation patient treated with 30 mg of edoxaban. (1) Edoxaban, (2) 4CA-EDX, (3) ND-EDX, and (4) 6-deuterium-labeled edoxaban as an internal standard.

Figure 3. Plasma concentrations of (A) edoxaban, (B) 4CA-EDX, and (C) ND-EDX in 20 patients with atrial fibrillation just before dosing on day 3 or later.

Edoxaban dose, 15 mg, cross; 30 mg, circle; and 60 mg, triangle.

Figure 4. Variations in dose and body weight-corrected plasma concentrations of (A) edoxaban, (B) 4CA-EDX, and (C) ND-EDX and metabolic ratios of edoxaban to (D) 4CA-EDX and (E) ND-EDX in atrial fibrillation patients.

Edoxaban dose, 15 mg, cross; 30 mg, circle; and 60 mg, triangle.

Table 1. Intra- and inter-day accuracy and imprecision of edoxaban and its metabolites in human plasma

Analyte	Theoretical value (ng/mL)	Intra-day (n = 6)			Inter-day (n = 6)		
		Mean ± SD (ng/mL)	Accuracy (%)	RSD (%)	Mean ± SD (ng/mL)	Accuracy (%)	RSD (%)
Edoxaban	1.6	1.56 ± 0.10	97.7	6.3	1.68 ± 0.20	104.8	11.8
	32	28.6 ± 0.7	89.4	2.4	28.6 ± 1.0	89.6	3.6
	128	140 ± 3	109.2	2.1	132 ± 4	103.3	2.7
4CA-EDX	0.6	0.677 ± 0.057	112.8	8.4	0.625 ± 0.063	104.2	10.1
	12	10.3 ± 0.4	85.9	4.2	10.4 ± 0.4	86.5	3.4
	48	46.7 ± 0.9	97.3	2.0	46.5 ± 1.8	96.9	3.8
ND-EDX	0.15	0.137 ± 0.018	91.2	13.3	0.150 ± 0.013	99.9	8.8
	3	3.08 ± 0.27	102.7	8.8	2.88 ± 0.25	95.9	8.6
	12	11.0 ± 0.9	91.3	8.6	11.4 ± 1.0	95.1	8.3

SD: standard deviation, RSD: relative standard deviation

Figure 1

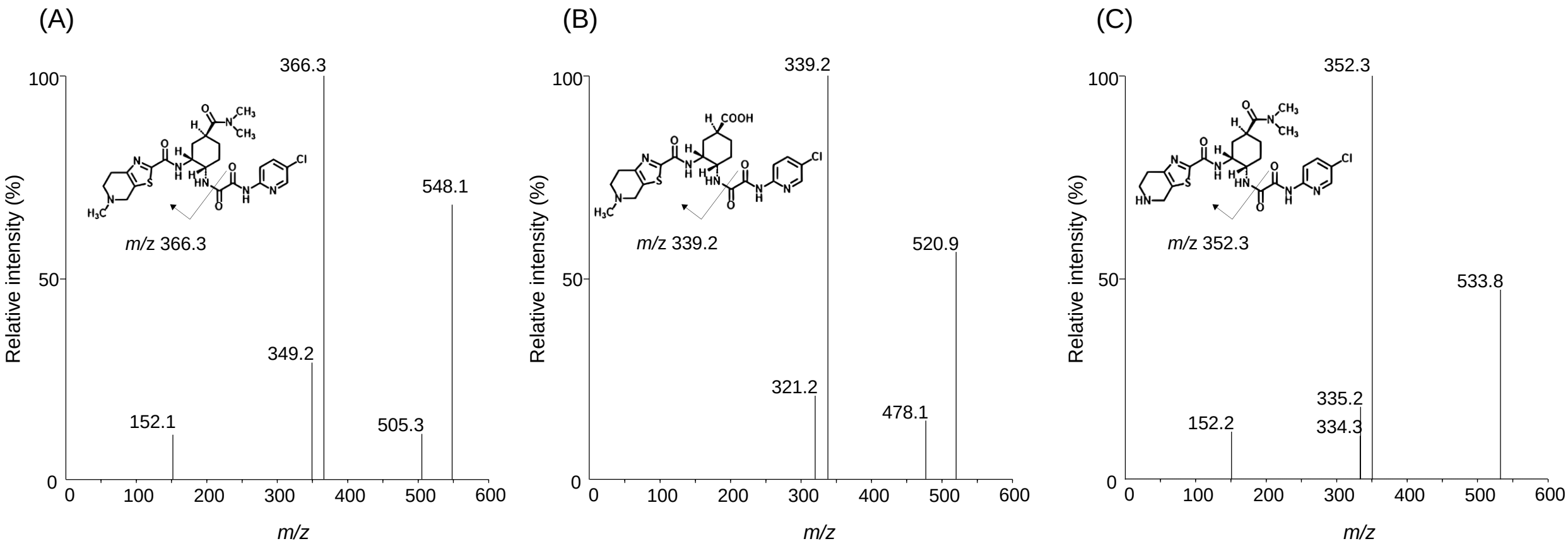

(A)
366.3
548.1
349.2
152.1
505.3
m/z 366.3
Relative intensity (%)
m/z
(B)
339.2
520.9
321.2
478.1
m/z 339.2
Relative intensity (%)
m/z
(C)
352.3
533.8
335.2
334.3
152.2
m/z 352.3
Relative intensity (%)
m/z

Figure 2

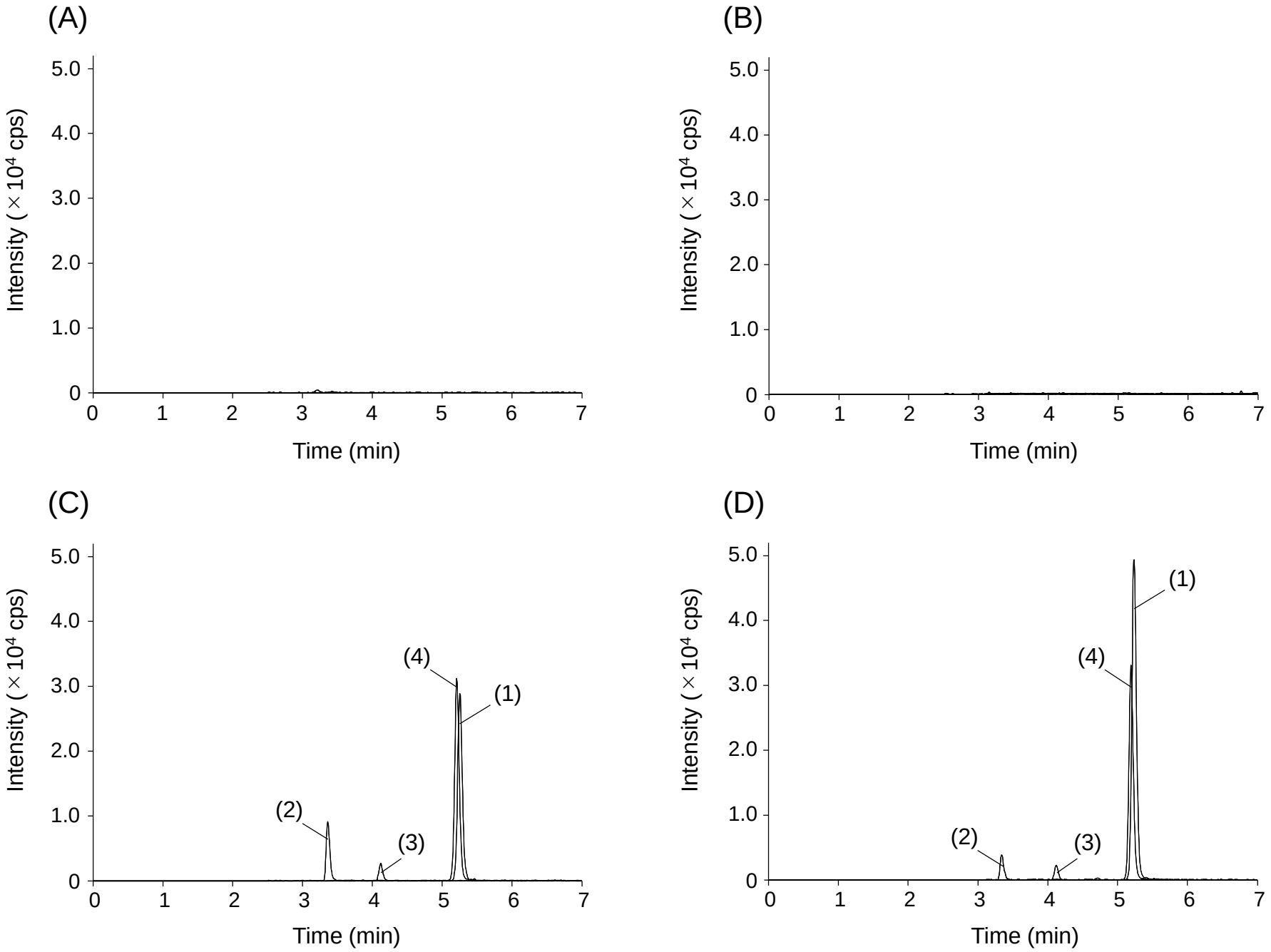


Figure 3

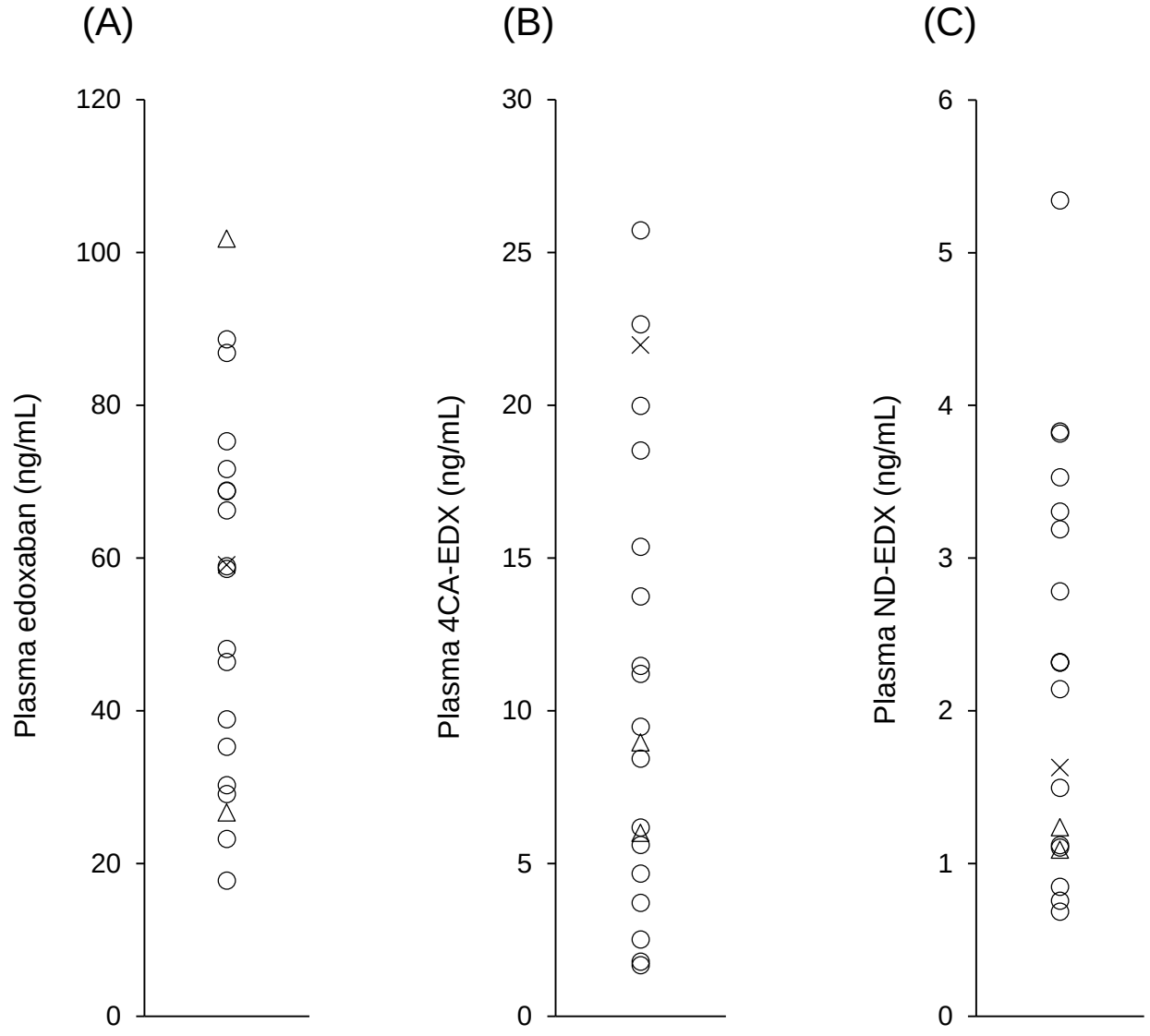
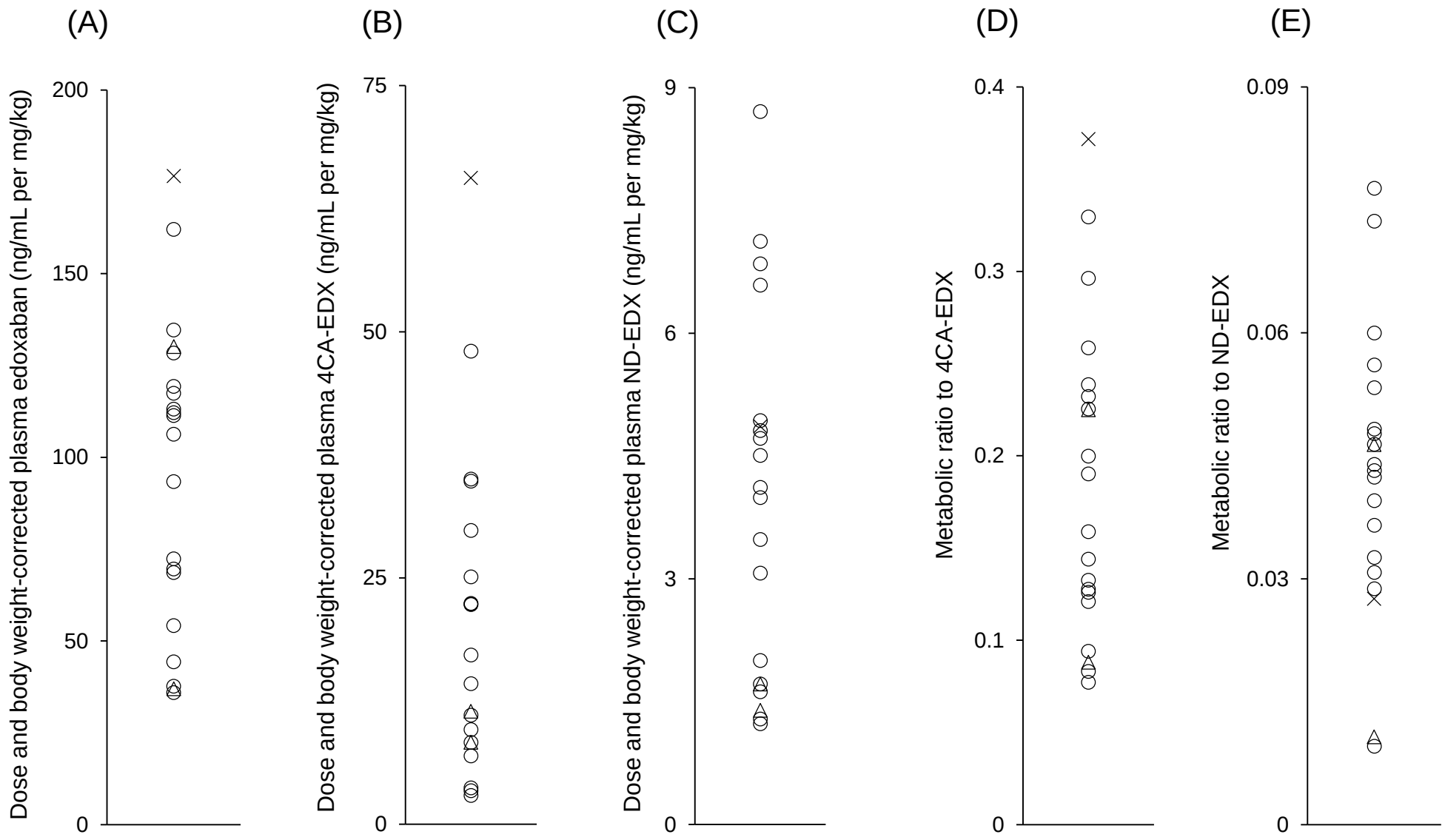

(A)
(B)
(C)
Plasma edoxaban (ng/mL)
Plasma 4CA-EDX (ng/mL)
Plasma ND-EDX (ng/mL)
120
100
80
60
40
20
0
30
25
20
15
10
5
0
6
5
4
3
2
1
0

Figure 4


(A)
(B)
(C)
(D)
(E)
Dose and body weight-corrected plasma edoxaban (ng/mL per mg/kg)
Dose and body weight-corrected plasma 4CA-EDX (ng/mL per mg/kg)
Dose and body weight-corrected plasma ND-EDX (ng/mL per mg/kg)
Metabolic ratio to 4CA-EDX
Metabolic ratio to ND-EDX
200
150
100
50
0
75
50
25
0
9
6
3
0
0.4
0.3
0.2
0.1
0
0.09
0.06
0.03
0