

Original Article



Bioanalytical methods for the detection of duloxetine and thioctic acid in plasma using ultra performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS)

Zhuodu Wei ¹, Hyeon-Cheol Jeong ¹, Ye-Ji Kang ^{1†}, Jaesang Jang ², Myoung-Hwan Kim ³, and Kwang-Hee Shin ^{1*}

¹College of Pharmacy, Research Institute of Pharmaceutical Sciences, Kyungpook National University, Daegu 41566, Korea

²Korea United Pharm. Inc., Seoul 06116, Korea

³Preclinical Research Center, NDIC Inc., Hwaseong 18469, Korea

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*Correspondence to

Kwang-Hee Shin

College of Pharmacy, Research Institute of Pharmaceutical Sciences, Kyungpook National University, 80 Daehak-ro, Buk-gu, Daegu 41566, Korea.

Email: kshin@knu.ac.kr

[†]Present address: Bacterial Disease Division, Animal and Plant Quarantine Agency, Gimcheon 39660, Korea.

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ORCID iDs

Zhuodu Wei

<https://orcid.org/0000-0003-4344-6427>

Hyeon-Cheol Jeong

<https://orcid.org/0000-0002-3500-6694>

Ye-Ji Kang

<https://orcid.org/0000-0002-1054-4963>

Jaesang Jang

<https://orcid.org/0000-0002-8377-9843>

Myoung-Hwan Kim

<https://orcid.org/0000-0003-3038-8053>

Kwang-Hee Shin

<https://orcid.org/0000-0002-0915-2700>

ABSTRACT

Duloxetine and thioctic acid (TA) are standard drugs for treating diabetic neuropathy, a primary complication associated with diabetes. In this study, ultra performance liquid chromatography coupled with tandem mass spectrometry methods was successfully developed and validated for quantifying duloxetine and TA in biological samples. The protein precipitation method was used to extract duloxetine, TA and their internal standards from beagle dog plasma. A Hypersil Gold C18 column (150 × 2.1 mm, 1.9 μm) was used for the experiment. Isocratic elution with 0.1% formic acid in acetonitrile (A) and 0.1% formic acid (B) was used for duloxetine, whereas a gradient elution with 0.03% acetic acid (A) and acetonitrile (B) was used for TA. The validated parameters included linearity, sensitivity, accuracy, precision, selectivity, matrix effect, stability, and recovery under different conditions. The linear ranges of the calibration curves for duloxetine and TA were 5–800 ng/mL and 5–1,000 ng/mL, respectively. An intra- and inter-run precision of ± 15% can be observed in all quality control samples. These methods were successfully used for pharmacokinetics (PKs) studies in beagle dogs to compare PK differences in a fixed-dose combination including duloxetine and TA and co-administration of the 2 drugs.

Keywords: Duloxetine; Thioctic Acid; Liquid Chromatography; Mass Spectrometry

INTRODUCTION

The peripheral nerves can be damaged in diabetic neuropathy as the body maintains a hyperglycemia for long periods [1]. Long-term hyperglycemia occurs in diabetic patients because of the clogging of capillaries delivering nutrients into the nervous system. A continuous increase in toxic metabolites, including advanced glycation end-products, further damages the nerve cells [2,3].

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Conflict of Interest

- Authors: Jaesang Jang is a paid employee at the Korea United Pharm. Inc., Korea. The other authors declare that they have no conflicts of interest.
- Reviewers: Nothing to declare
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Author Contributions

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Duloxetine and thioctic acid (TA) are common therapeutic options for painful diabetic neuropathy. Duloxetine, an inhibitor of serotonin and norepinephrine re-uptake, is used to relieve peripheral neuropathic pain and to ameliorate symptoms of depression caused by sustained pain [4]. TA, an antioxidant, is capable of reducing inflammation in nerves and relieving the pain [5]. Many prospective placebo-controlled studies based on its antioxidant mechanism have been investigated and found that TA has a powerful effect in relieving pain in patients with painful diabetic neuropathy [6-8].

Several methods have been developed for quantifying duloxetine and TA; however, these methods have several disadvantages. Methods for duloxetine analysis include those using liquid chromatography–tandem mass spectrometry (LC-MS/MS) [9-12], gas chromatography–mass spectrometry [13,14], and high performance liquid chromatography–UV detection (HPLC-UV detection) [15]. The drawbacks of these methods are long run times (5–6 minutes) [9,12], intricate derivatization processes or extraction steps [13,14], and a high limit of quantification (2 µg/mL) [15]. HPLC is considered the most appropriate method for detecting TA [16]. Some methods have been reported to detect TA by LC [16-23]. The disadvantages of the previously reported methods include elaborate solid-phase extraction or derivatization steps [20,23], long run times (5–15 minutes) [16,17,19], and relatively poor sensitivity (0.13–5 µg/mL) [18,21,22]. Considering the limitations of these previous methods, a faster, simpler, and more sensitive detection method for duloxetine and TA is needed.

The main purpose of the current study was to establish and validate a fast, sensitive and reliable ultra performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS) method for the determination of duloxetine and TA in analytical samples and to employ the established methods for a pharmacokinetics (PKs) study of beagle dog plasma.

METHODS

Chemicals, reagents, and other materials

Reference standards for duloxetine and TA were provided by the Korea United Pharm., Inc. (Seoul, Korea). The purity of duloxetine was 100% and that of TA was 99.6%. Internal standard (IS) of duloxetine, duloxetine- d_3 , was purchased from Sigma-Aldrich (St. Louis, MO, USA) with the purity of 99.17%; the IS of TA, TA- d_5 , was purchased from Toronto Research Chemicals (Ontario, Canada) with a purity of 99.9%. Acetonitrile, methanol, and water were of LC-MS grade and purchased from Merck (Darmstadt, Germany). Formic acid and acetic acid were obtained from Sigma-Aldrich.

Preparation of standards and quality control (QC) samples

Methanol (100%) was used to prepare for the duloxetine and duloxetine- d_3 stock solutions (1 mg/mL) and working solutions (50, 100, 200, 500, 1,000, 2,000, 4,000, and 8,000 ng/mL for duloxetine and 400 ng/mL for duloxetine- d_3). The concentrations of the QC working solutions were 150 ng/mL (low quality control, LQC), 1,500 ng/mL (middle quality control, MQC), and 6,400 ng/mL (high quality control, HQC) for duloxetine. For TA, and TA- d_5 , methanol:water (1:1, v/v) was used to prepare stock solutions (1 mg/mL) and working solutions (50, 100, 200, 500, 1,000, 2,000, 5,000, and 10,000 ng/mL for TA and 20 µg/mL for TA- d_5). The concentration of QC working solutions for TA were 150 ng/mL (LQC), 1,000 ng/mL (MQC), and 8,000 ng/mL (HQC). A dilution factor of 10 was used when preparing for calibrations and QC samples from the working solutions.

Sample preparation

A one-step protein precipitation process was used to extract duloxetine from beagle dog plasma. Firstly, 100 μL of plasma and 50 μL of IS solution (400 ng/mL) were placed in a 1.5 mL tube and mixed for 30 seconds. The protein was precipitated by adding 450 μL of 100% acetonitrile, followed by centrifugation (13,000 rpm, 10 minutes, 4°C). Finally, 200 μL of the supernatant was placed into a new tube, and 4 μL of the supernatant was analyzed using the UPLC. For analyzing TA, 100 μL of plasma sample and 300 μL of acetonitrile containing IS (TA- d_5 , 200 ng/mL) were added, followed by vortexing for 5 minutes and centrifugation (16,300 g, 15 minutes, 4°C). Next, 350 μL of the supernatant was dried under a nitrogen atmosphere at 40°C. A mobile phase (100 μL , acetonitrile:0.03% acetic acid = 40:60, v/v) was used to reconstitute the residue, following vortexing, mixing, and centrifugation as above. Subsequently, 5 μL of the solution was analyzed.

Liquid chromatography and mass spectrometric conditions

Different UPLC-MS/MS instruments and conditions were used to analyze the concentration of duloxetine and TA. For duloxetine and duloxetine- d_5 , the UPLC system included an Acquity™ UPLC® (Waters, Milford, MA, USA), autosampler, binary pump, and a Hypersil Gold C18 column (150 \times 2.1 mm, 1.9 μm) (Thermo Fisher Scientific, Carlsbad, CA, USA). An isocratic elution (acetonitrile with 0.1% formic acid [A]:0.1% formic acid [B] = 75:25, v/v) at a flow rate of 0.3 mL/min was employed to separate duloxetine and IS from the sample. The running time for each sample of duloxetine was 3 minutes. Sample vials were placed in an autosampler and maintained at 10°C. Quantification was performed using a XEVO TQ-MS (Waters) with electrospray ionization (ESI) in positive ion mode and multiple reaction monitoring (MRM) mode for duloxetine. The mass spectra of duloxetine and the IS in the ESI positive mode are shown in **Fig. 1**. The optimized source-dependent parameters for duloxetine included a desolvation temperature of 400°C, desolvation gas flow rate of 900 L/h, and a collision energy of 10 eV for duloxetine and 6 eV for IS. Data were processed using the Masslynx software, version 4.1 (Waters). For TA and TA- d_5 , a Vanquish UPLC system and a Hypersil Gold C18 column (150 \times 2.1 mm, 1.9 μm) (Thermo Fisher Scientific) were used with a gradient elution containing 0.03% acetic acid (A) and 100% acetonitrile (B). The gradient elution was performed as follows: 0–0.5 minutes, 40% B; 0.5–1.5 minutes, 40–90% B; 1.5–2.3 minutes, 90% B; 2.3–2.5 minutes, 90–40% B, and maintained 40% B to 5.5 minutes (0.3 mL/min). Sample vials were placed in an autosampler (10°C). Quantification of TA and IS were quantified using a Thermo TSQ Vantage with ESI in the negative mode. The selected reaction monitoring (SRM) mode was chosen to detect TA and TA- d_5 , and the mass spectra are also shown in **Fig. 1**. The optimized parameters for the analysis of TA included a capillary temperature of 350°C, sheath gas pressure of 50 Arb, and collision energies of 12 V for TA and 13 V for IS. Data analyses were conducted using Xcalibur version 4.1 software (Thermo Fisher Scientific).

Bioanalytical method validation

Method validation test items included calibration curve linearity, sensitivity, selectivity, accuracy, precision, matrix effect, re-injection stability, stability of standard, short-term stability, freeze-thaw stability, processed sample stability, and recovery. The validation process was performed based on the “Guidance for Industry Bioanalytical Method Validation” from the U.S. Food and Drug Administration and “Guideline on Bioanalytical Method Validation” published by the Ministry of Food and Drug Safety in Korea [24,25].

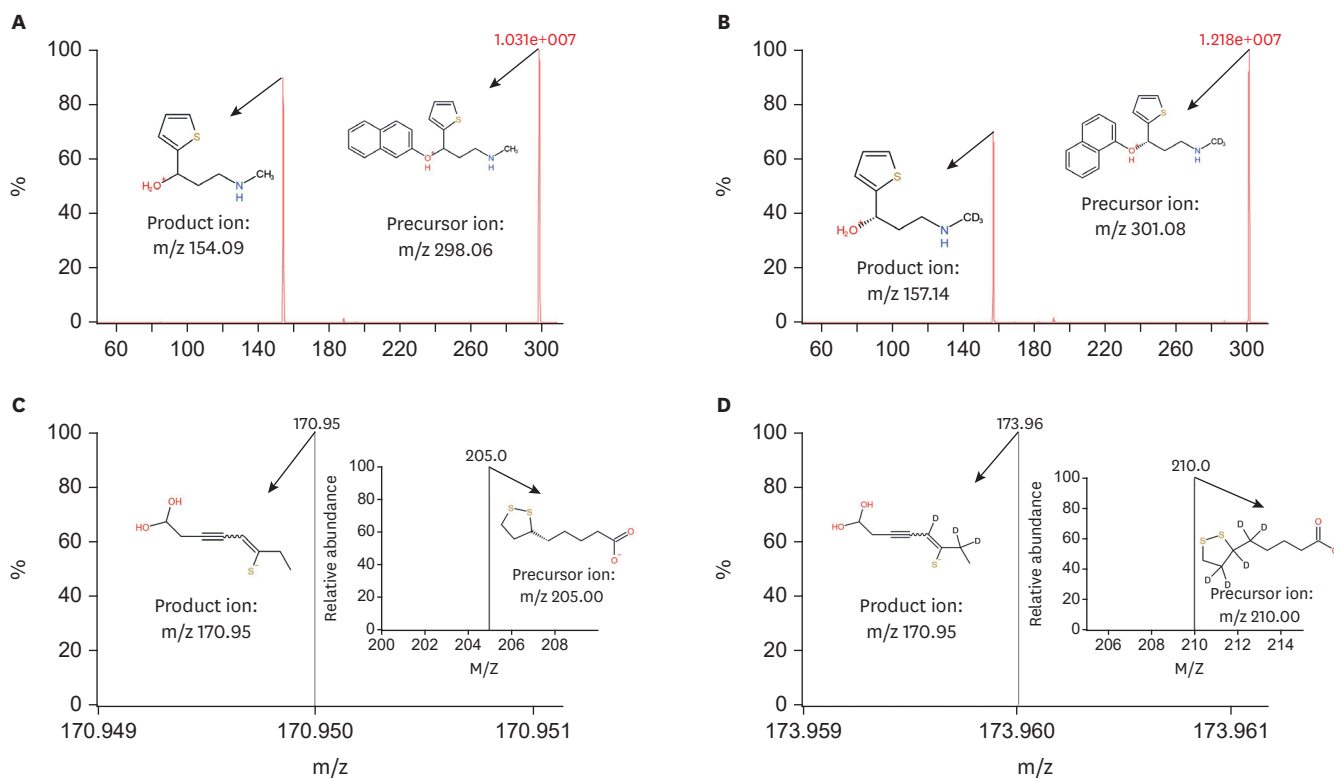


Figure 1. Mass spectra of (A) duloxetine and (B) internal standard (duloxetine- d_3) in ESI positive mode, and (C) thioctic acid and (D) its internal standard (thioctic acid- d_3) in ESI negative mode. ESI, electrospray ionization.

Linearity and sensitivity

Linearity was verified using a calibration curve prepared from the samples of duloxetine and TA. The calibration curve contained a double blank sample (no reference standard, no IS), a blank sample (only containing IS), and 8 standard samples over a concentration range of 5–800 ng/mL concentration range for duloxetine and 5–1,000 ng/mL for TA. Linearity was deemed acceptable if the correlation coefficient (R) was greater than 0.9950. Sensitivity was evaluated using the signal-to-noise ratio (S/N ratio), which compared the signal level of the lower limit of quantification (LLOQ) to that of the double blank (background noise). The assessment criterion for sensitivity was that the S/N ratio of LLOQ should be more than 10 times higher than that of the double blank samples.

Accuracy and precision

Accuracy and precision were determined using 4 QC samples (LLOQ, LQC, MQC, and HQC). To evaluate the intra-run precision, each batch and concentration was repeated 5 times. The inter-run precision was assessed by repeating the analysis of the 3 batches. The accuracy was deemed acceptable if the measured concentration was less than $\pm 15\%$, except $\pm 20\%$ for LLOQ. For precision, all test samples should meet the criteria of $\pm 15\%$ coefficient of variation (CV), except $\pm 20\%$ at LLOQ.

Selectivity and matrix effects

A selectivity test was performed using beagle dog matrix from 6 individual resources. Six sets of double blank samples and LLOQ samples were pre-treated and analyzed. The matrix effect was determined by the impact of interference on individual plasma samples by evaluating

the peak area ratio of the analyte and IS. Equation (Eq. 1) was applied to calculate the matrix effect. The selectivity and matrix effects were measured using LQC and HQC samples prepared from 6 individual plasma samples. Acceptance criteria for the selectivity and matrix effects were values within $\pm 15\%$ CV.

$$\text{Matrix Effect} = A/B \times 100 \text{ (Eq. 1)}$$

where A represents the standard deviation (SD) of the area ratio spiked at each of the 2 concentrations for the 6 plasma samples, and B is the mean of the area ratio spiked at each of the 2 concentrations for 6 plasma samples.

Stability and recovery

Stability evaluation items included stability at room temperature (short-term stability), stability following repeated freezing and thawing (freezing and thawing stability), storage stability in an autosampler (stability of pretreated samples), and stability of standard stock solutions. The short-term stock and working solution stability were determined after storage at room temperature (20–25°C) for 12 hours. Freeze-thaw cycles at both room and storage temperatures were repeated for 3 cycles to assess the freeze-thaw stability. To determine the stability of the processed samples, they were stored in an autosampler (10°C) for 24 hours. Stability tests were conducted for each of the LQC and HQC samples. The recovery was evaluated by comparing the test samples obtained by adding the working solutions after evaporation with the reference samples, which were prepared using the original preparation method. A recovery test was performed for each analyte at 3 different concentrations (LQC, MQC and HQC). The % change in all test samples should be within 15%.

Application of UPLC-MS/MS in beagle dog plasma samples

The animal study was conducted at KPC Co. Ltd (Gwangju, Korea) and was approved by the Institutional Animal Care and Use Committee of KPC Co. Ltd. (current name: NDIC Inc.). Gyeonggi Province, Korea (KPC-IACUC, approval No. P193042). Twelve healthy male beagle dogs (*Canis lupus familiaris*) (mean \pm SD of weight, 9.9 ± 0.9 kg) were randomly divided into 2 treatment groups. Test group: administered a fixed-dose combination. Reference group: co-administered of 2 drugs. The dogs were fasted overnight prior to blood collection. Whole blood was collected from the jugular vein into EDTA-K₂ tubes at pre-dose (0 hour) and 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6, 8 and 12 hours after co-administration of one capsule Cymbalta® (67.3 mg of duloxetine hydrochloride [60 mg as duloxetine]) and one tablet Dexid® (480 mg of R-TA tromethamine [300 mg as R-TA]) (reference group), or a fixed-dose single tablet (67.3 mg of duloxetine hydrochloride [60 mg as duloxetine]; 480 mg of R-TA tromethamine [300 mg as R-TA]) (test group). Plasma samples were obtained by centrifugating the whole blood for 10 minutes at 4,000 rpm. The obtained plasma samples were stored at -80°C until analysis. The concentrations of duloxetine and TA in the samples were measured using the prepared calibration curve. Concentration–time profiles and PK parameters were obtained from non-compartmental analysis (NCA) in R (version 4.0.2, R: A language and environment for statistical computing; R Foundation for Statistical Computing, Vienna, Austria; URL: <https://www.R-project.org/>) using the pkr package (Pharmacokinetics in R; URL: <https://cran.r-project.org/package= pkr>). The peak plasma concentration (C_{\max}), the time to reach C_{\max} (T_{\max}), area under the plasma concentration versus time curve until the last quantifiable time point (AUC_{last}) (linear-up log-down), clearance (CL), and their ratios (test/reference) were calculated. Differences between the test and reference groups were assessed using the Mann-Whitney U test (SPSS software version 26; SPSS Inc., Chicago, IL, USA).

RESULTS

Method development

As the difference in sensitivity of the detected peaks using our method was insignificant compared to that of the peaks detected using the liquid-liquid extraction method with methyl tert-butyl ether and ethyl acetate, the extraction of duloxetine and TA samples was processed using one-step protein precipitation with acetonitrile. Duloxetine and duloxetine- d_3 (IS) achieved the optimal peak shape and sensitivity in (A) acetonitrile with 0.1% formic acid and (B) 0.1% formic acid in deionized water. Using (A) 0.03% acetic acid and (B) 100% acetonitrile, TA and TA- d_5 (IS) using in the smallest background noise, best peak shape, and greatest possibility of reproduction compared to the other solvents. The chromatographic conditions were selected based on the physicochemical properties of duloxetine and TA. If the duloxetine analysis, if the mobile phases were acetonitrile and water, a high background noise was observed in chromatography of duloxetine. As duloxetine is a strong base (pKa 9.7), the degree of extraction from plasma can be improved by adding 0.1% formic acid to the water or acetonitrile [26]. The ionization efficiency of duloxetine was increased with a high organic solvent ratio (acetonitrile with 0.1% formic acid and 0.1% formic acid, 75:25, v/v). For TA analysis, 0.03% acetic acid and acetonitrile as the mobile phase were favorable, with very low background noise. As shown in **Fig. 1**, duloxetine and duloxetine- d_3 were detected using the positive mode $[M+H]^+$ in the MRM model. The precursor ion m/z values of duloxetine and duloxetine- d_3 were 298.06 and 301.08, respectively. The naphthalene structure of duloxetine and duloxetine- d_3 was cleaved in the ESI, forming the product ion of duloxetine and duloxetine- d_3 . The product ion m/z of duloxetine and duloxetine- d_3 were 154.09 and 157.14, respectively. TA and TA- d_5 were analyzed using the negative model $[M-H]^-$ in SRM, with m/z values of 205.00 and 210.00 for the TA and TA- d_5 precursor ions, respectively. The product ions were at m/z 170.95 and m/z 173.96 for TA and TA- d_5 , respectively.

Linearity and sensitivity

Linearity ranges for plasma duloxetine and TA were established at 5–800 ng/mL ($r \geq 0.997$) and 5–1,000 ng/mL ($r \geq 0.9995$), respectively. Linear regression was performed using the equation, $y = ax + b$. The weighting factors were set to $1/x^2$ and $1/x$ for duloxetine and TA, respectively. The developed method satisfied the acceptance criterion with a correlation coefficient (r) should be more than 0.997 and the detailed results are presented in **Table 1**. The S/N ratios of the LLOQ samples for duloxetine and TA exceeded 10 when comparing the signal reaction of the analyte of the LLOQ and that of the double blank sample, which indicates satisfactory sensitivity. Representative chromatograms of the double blank sample and the LLOQ are shown in **Fig. 2**.

Table 1. Slope, intercept, and correlation coefficient (r) of the calibration curves for duloxetine and thioctic acid

Compounds	Duloxetine	Thioctic acid
Concentration ranges (ng/mL)	5–800	5–1,000
Slope		
Mean \pm SD	1.983 \pm 0.153	0.001 \pm 0.0001
%CV	8.60	5.68
Intercept		
Mean \pm SD	0.999 \pm 0.001	0.999 \pm 0.0001
%CV	0.045	0.01
Correlation coefficient (r)	≥ 0.997	≥ 0.9995

SD, standard deviation; CV, coefficient of variation.

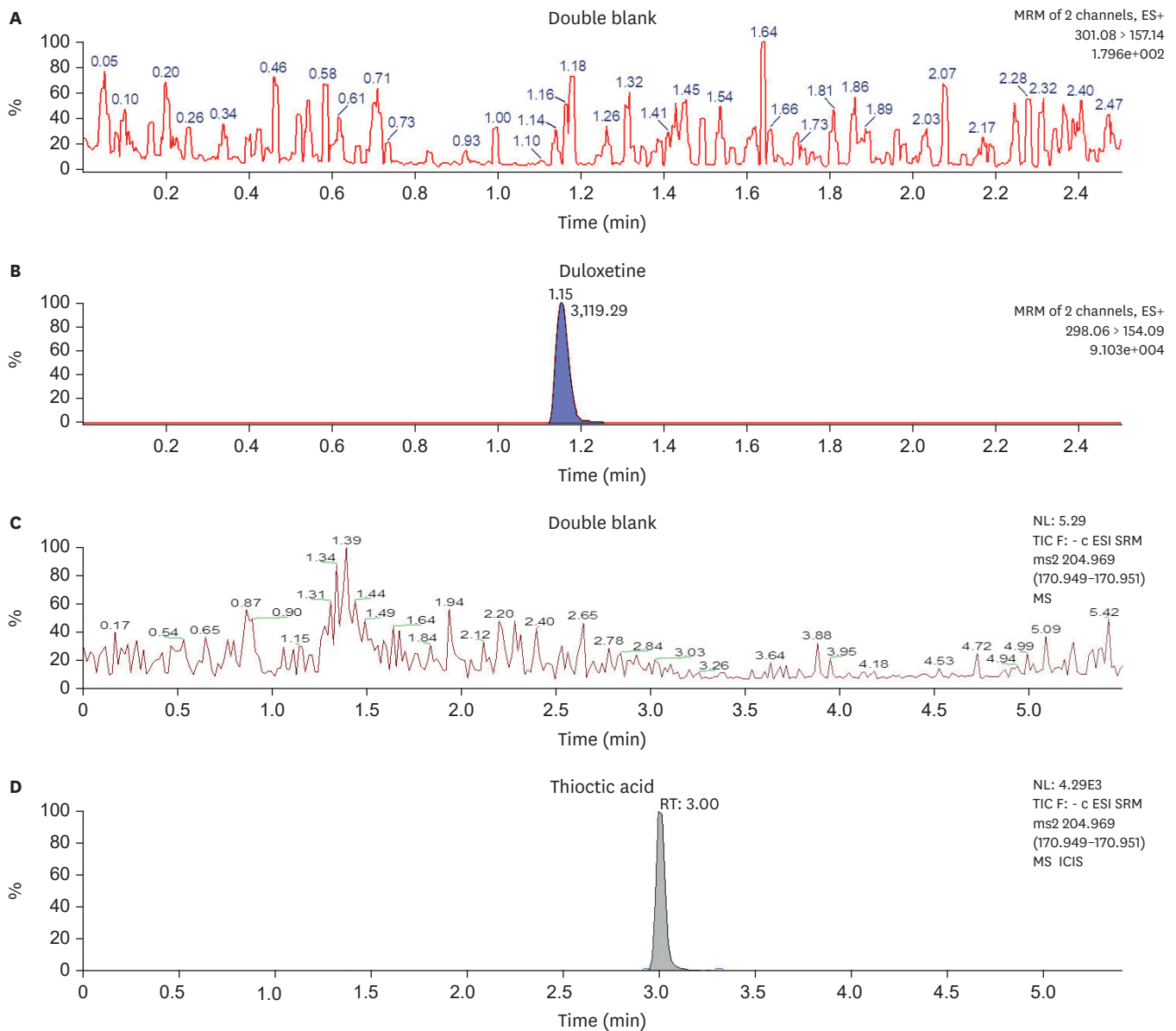


Figure 2. Representative chromatograms of double blank beagle dog plasma containing (A) duloxetine and (B) its LLOQ samples, and (C) thioctic acid and (D) its LLOQ samples.

LLOQ, lower limit of quantification; NL, normalization level; TIC, total ion chromatogram; MS, mass spectrometry; ICIS, interactive chemical information system; RT, retention time; MRM, multiple reaction monitoring; ESI, electrospray ionization; SRM, selected reaction monitoring.

Accuracy and precision

Accuracy and precision were assessed with 3 independent analysis runs, 4 QC levels in each run (LLOQ, L, M, and HQC) and 5 replicates at each QC level. Representative chromatograms for duloxetine and TA in beagle dog plasma after the administration of a fixed-dosed combination tablet are shown in **Fig. 3**. The assessment results for duloxetine and TA are exhibited in **Table 2**. The accuracy of duloxetine was between 96.36% and 108.44%, and the precision (%CV) was between 0.11–7.73. For TA, the accuracy was between 98.00% and 107.45%, and the intra- and inter-batch precisions were within 0.10–7.35. The measured concentrations for both drugs met the pre-specified criteria ($\pm 15\%$ of nominal concentrations or $\pm 15\%$ CV, except $\pm 20\%$ at LLOQ).

Table 2. Intra- and inter-day precision and accuracy data for the duloxetine and thioctic acid assays in beagle dog plasma

Concentrations	Duloxetine				Thioctic acid			
	LLOQ (5 ng/mL)	LQC (15 ng/mL)	MQC (150 ng/mL)	HQC (640 ng/mL)	LLOQ (5 ng/mL)	LQC (15 ng/mL)	MQC (100 ng/mL)	HQC (800 ng/mL)
Accuracy (%)								
Batch 1	105.04	108.44	104.59	106.49	98.00	98.58	100.93	100.38
Batch 2	100.96	99.15	98.61	101.83	101.83	107.45	100.79	100.94
Batch 3	96.36	105.23	100.01	100.72	106.83	98.28	99.68	101.42
Inter-batch	100.79	104.27	101.07	103.02	102.22	101.44	100.47	100.92
Precision (%CV)								
Batch 1	7.73	2.14	2.27	1.65	3.95	0.96	1.14	0.64
Batch 2	4.89	2.66	1.58	2.46	1.89	3.21	1.98	0.82
Batch 3	0.11	5.40	3.97	1.71	0.10	1.72	2.32	0.38
Inter-batch	7.25	3.68	3.10	3.10	7.35	1.83	0.73	0.73

LLOQ, lower limit of quantification; LQC, low quality control; MQC, middle quality control; HQC, high quality control; CV, coefficient of variation.

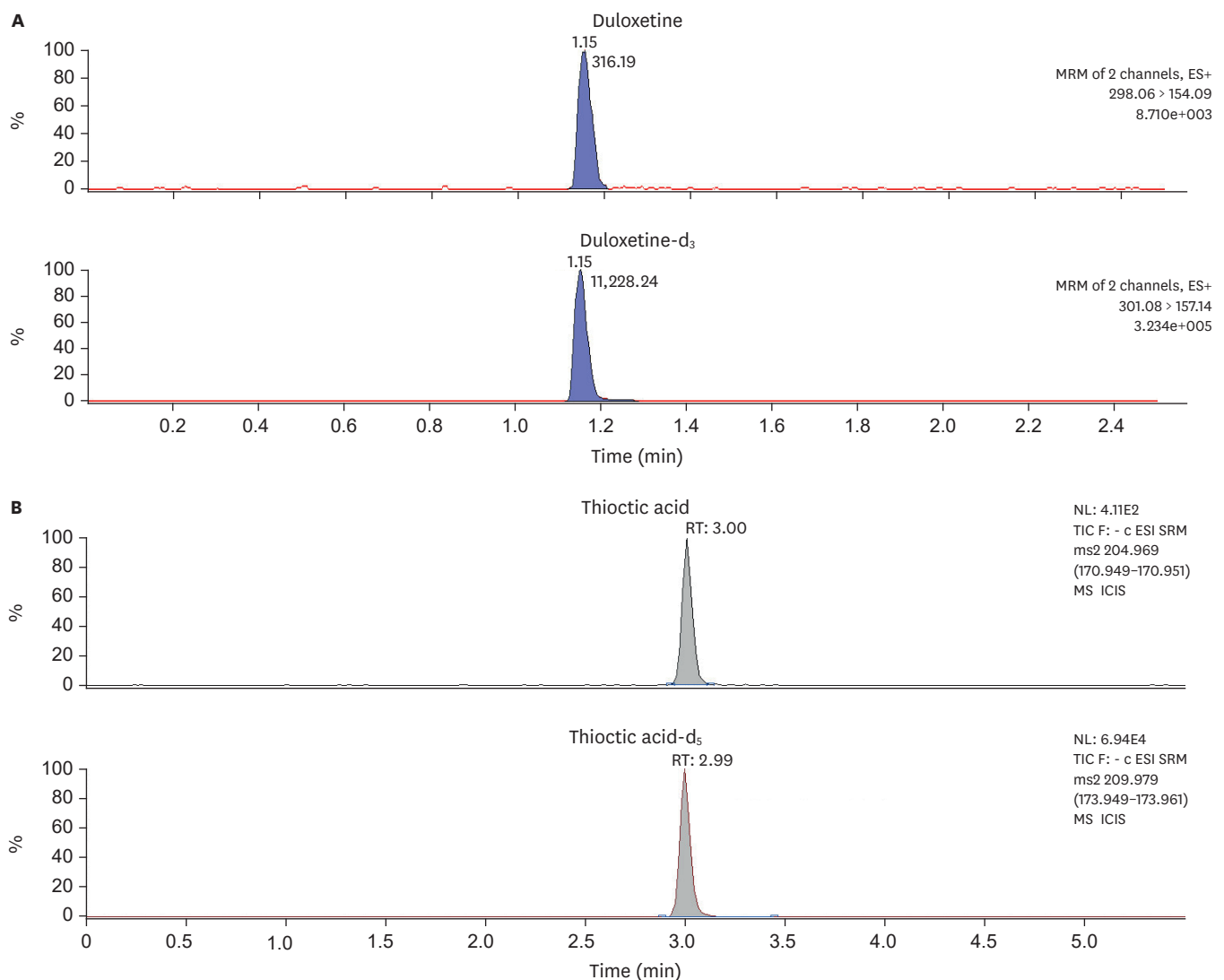


Figure 3. Representative chromatograms of (A) duloxetine and IS (duloxetine-d₃), and (B) thioctic acid and IS (thioctic acid-d₅) in beagle dog plasma after administration of a fixed-dose combination tablet of duloxetine and thioctic acid. IS, internal standard; NL, normalization level; TIC, total ion chromatogram; MS, mass spectrometry; ICIS, interactive chemical information system; RT, retention time; MRM, multiple reaction monitoring; ESI, electrospray ionization; SRM, selected reaction monitoring.

Table 3. Matrix effects in beagle dog plasma (n = 6)

Number	Duloxetine		Thioctic acid	
	LQC (15 ng/mL)	HQC (640 ng/mL)	LQC (15 ng/mL)	HQC (800 ng/mL)
1	34.13	1,221.76	0.015	0.892
2	28.53	1,196.35	0.016	0.900
3	27.82	1,180.54	0.016	0.907
4	27.84	1,224.56	0.017	0.914
5	29.47	1,179.33	0.016	0.912
6	28.27	1,217.51	0.016	0.883
%CV	8.26	1.72	4.15	1.34

All values are represented as peak area ratios.

LQC, low quality control; HQC, high quality control; CV, coefficient of variation.

Selectivity and matrix effects

Selectivity tests were performed using blank beagle dog plasma from 6 resources. Six sets of double blank samples and LLOQ samples were pretreated and analyzed. Spiked samples were within $\pm 20\%$ LLOQ and the IS response of the blanks did not exceed 5% of the averages IS responses in the calibrators and QCs. The CVs of LQC and HQC obtained from 6 different individuals' plasma were 8.26% and 1.72% for duloxetine. The CVs of LQC and HQC for TA samples were 4.15% and 1.34%, respectively. These results revealed that the %CV at each concentration (LQC and HQC) met the criterion and was less than 15% (**Table 3**).

Stability and recovery

The samples were analyzed under diverse conditions to determine their stability (**Table 4**). The % change of LQC and HQC samples proceed in the 10°C autosampler for 24 hours for both duloxetine and TA was within $\pm 15\%$. The % change of LQC and HQC samples in the 3 freeze-thaw cycles was within $\pm 15\%$, except for the LQC samples of TA slightly exceeding the range (-21.16%), which indicated that TA may be unstable in plasma after 3 freeze-thaw cycles. Both the short-term stock solution and working solutions remained stable at room temperature for 12 hours. The results obtained by repeating the test at least thrice for each concentration were evaluated. At each concentration, the precision, and those CV% were also within 15%, indicating that the recovery of duloxetine and TA met the acceptance criterion.

PK study

The plasma duloxetine and TA concentration–time profiles for the test group (fixed-dose combination of these 2 components) and the reference group (co-administration of separate doses) groups are shown in **Fig. 4**. The PK parameters calculated by NCA are presented in **Table 5**. For the analysis of duloxetine, the arithmetic mean \pm SD AUC_{last} was 115.12 ± 73.40 ng·h/mL for the test group and 141.77 ± 160.95 ng·h/mL for the reference group. The mean \pm SD C_{max} and median (min-max) T_{max} were 49.32 ± 27.14 ng/mL and 1.50 (0.50–2.50) h for the test group, respectively, whereas in the reference group, C_{max} and T_{max} were 67.54 ± 81.74 ng/mL and 2.00 (0.50–2.50) h, respectively. The results for TA indicated that the arithmetic mean AUC_{last} was $5,141.16 \pm 5,611.99$ ng·h/mL for the test group and $4,165.74 \pm 1,434.53$ ng·h/

Table 4. Stability of duloxetine and thioctic acid under various conditions at 2 concentrations (n = 3)

Concentrations	Duloxetine		Thioctic acid	
	LQC (15 ng/mL)	HQC (640 ng/mL)	LQC (15 ng/mL)	HQC (800 ng/mL)
Stability	% Change		% Change	
Processed sample stability at 10°C for 24 hr	-7.14	-0.69	1.93	1.19
Three freeze-thaw stability at -80°C	-14.07	-11.31	-21.16	-1.60
Short-term stability at room temperature for 12 hr	13.45	5.12	4.15	-4.66
Stock solution stability at room temperature for 12 hr	-5.75	-1.54	4.28	-4.66

LQC, low quality control; HQC, high quality control.

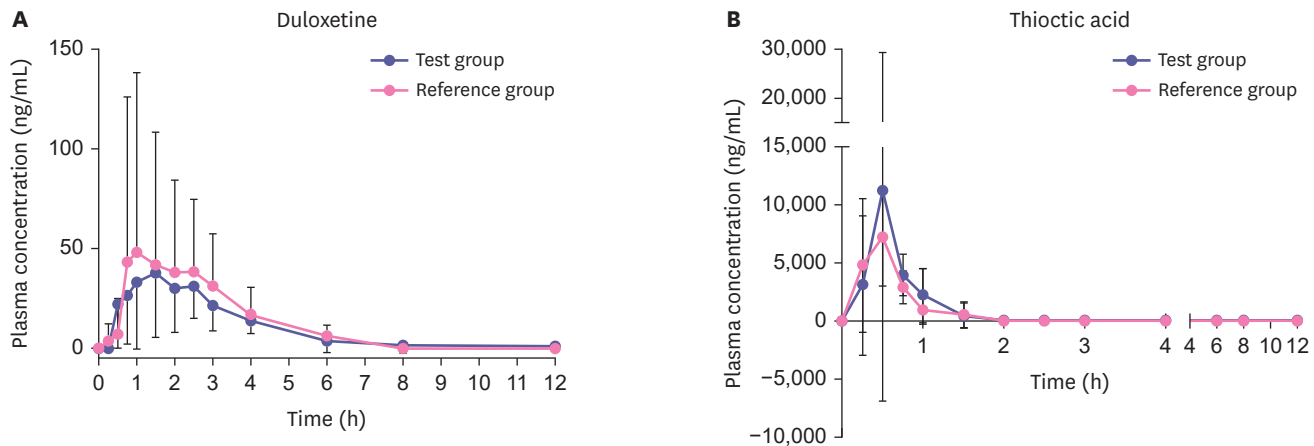


Figure 4. Plasma (A) duloxetine and (B) thioctic acid concentration–time profiles after administration of duloxetine (60 mg) and thioctic acid (480 mg) as separate doses (reference groups, pink circle), or as a fixed-dose combination tablet (test group, purple circle). Error bars indicate the standard deviation.

Table 5. Pharmacokinetic parameters of duloxetine and thioctic acid in the test and reference groups (n = 12)

Variables	Test (n = 6)	Reference (n = 6)	Ratio (test/reference)	p-value
Duloxetine				
C_{max} (ng/mL)	49.32 ± 27.14	67.54 ± 81.74	0.73 (–0.24–1.70)	0.818
T_{max} (hr)	1.50 (0.50–2.50)	2.00 (0.50–2.50)	-	0.818
AUC_{last} (ng·h/mL)	115.12 ± 73.40	141.77 ± 160.95	0.81 (–0.25–1.87)	0.818
CL/F (L/h)	387.03 ± 204.27	555.30 ± 312.39	-	0.352
Thioctic acid				
C_{max} (ng/mL)	12,923.58 ± 17,212.81	8,863.67 ± 4,815.53	1.46 (–0.64–3.56)	0.699
T_{max} (hr)	0.63 (0.50–1.00)	0.50 (0.25–1.00)	-	0.240
AUC_{last} (ng·h/mL)	5,141.16 ± 5,611.99	4,165.74 ± 1,434.53	1.23 (–0.18–2.65)	0.485
CL/F (L/h)	173.79 ± 108.12	125.62 ± 38.29	-	0.485

Data are presented as the mean ± standard deviation except for T_{max} as the median (min–max).

Test group, fixed-dose combination tablet of duloxetine and thioctic acid; reference group, co-administered duloxetine and thioctic acid as separate doses; C_{max} , peak plasma concentration; T_{max} , time to peak concentration; AUC_{last} , area under the plasma concentration vs. time curve from time 0 to the last quantifiable time point; CL, clearance.

mL for the reference group, respectively. The mean C_{max} and median T_{max} for the TA test group were 12,923.58 ± 17,212.81 ng/mL and 0.63 (0.50–1.00) h, respectively, whereas in the reference group, the C_{max} and T_{max} were 8,863.67 ± 4,815.53 ng/mL and 0.50 (0.25–1.00) h, respectively. The CL of duloxetine was 387.03 ± 204.27 L/h and 555.30 ± 312.39 L/h for the test and reference group, whereas the CL of TA was 173.79 ± 108.12 L/h and 125.62 ± 38.29 for the test and reference group, respectively.

DISCUSSION

In this study, quantitative bioanalytical methods were successfully established and validated for duloxetine and TA using a UPLC-MS/MS system. All the test parameters fulfilled the predefined criteria. A small plasma volume (0.1 mL) was used to prepare the samples for duloxetine and TA and a small injection volume (4 µL for duloxetine and 5 µL for TA) was used for the experiments. The analytical run time of 2.5 minutes for duloxetine and 5.5 minutes for TA was found to be relatively short. Excellent linearity ($r \geq 0.997$ over the range of 5–800 ng/mL for duloxetine and ≥ 0.9995 over the range of 5–1,000 ng/mL for TA) was also achieved. These results suggest that developed method is capable of accurately measuring the plasma concentrations of duloxetine and TA in the plasms of beagle dogs.

Although several methods have been proposed to measure the concentrations of duloxetine and TA in plasma, the procedures described here have distinct advantages. A recent article describing a validated method for measuring TA was published by Trivedi et al. [27]. In Trivedi's study [27], the bioanalytical methods to determine the concentrations of 5–1,000 ng/mL were developed and validated. The accuracy (%) and precision (%RSD) were 92.25–109.69% and 0.93–13.77%, respectively, in that study, whereas these were 98.00–107.45% and 0.10–7.35%, respectively, in our study. Compared to another recently validated method for the determination of duloxetine by Chen et al [9], in which gradient elution was used with a retention time of 3.15 minutes, the method developed here utilized a simple isocratic elution method with a rapid retention time (1.15 minutes), which saved time.

Our methods were employed to measure the concentration of duloxetine and TA and to compare the PK differences between 480 mg TA and 60 mg duloxetine as a fixed-dose combination and separated doses in 12 beagle dogs. Between the 2 treatment groups, the PK parameters (C_{max} , T_{max} , AUC_{last} , and CL/F) of the 2 drugs showed no significance ($p > 0.05$). The arithmetic ratios (test group/reference group) of C_{max} and AUC_{last} were 0.73 (–0.24–1.70) and 0.81 (–0.25–1.87) for duloxetine and 1.46 (–0.64–3.56) and 1.23 (–0.18–2.65) for TA, respectively. A relatively high deviation was observed between the reference and test groups, which may be the result of individual variability and small sample size. As shown in **Fig. 4**, the PK profiles of duloxetine and TA were statically similar.

A limitation of the current study was the relatively higher LLOQs of 5 ng/mL for both duloxetine and TA. Lower LLOQs (0.2, 0.5, and 0.05 ng/mL) of both drugs have been reported [10,19,28]. An LLOQ of 0.05 ng/mL for duloxetine was reported by Gajula et al. [10]. However, a simple one-step precipitation method was applied in the current study and the results showed a similar precision and accuracy (0.11–7.73% and 96.36–108.44%, respectively) as compared to that of Gajula et al. [10] (0.82–9.81% and 92.23–111.39%, respectively) in which a complex solid-phase extraction technique was used. Kobayashi et al. [19] reported an LLOQ of 0.5 ng/mL for TA. However, the run time for one sample was 30 minutes, which was substantially longer than that of the present study (5.5 minutes). Therefore, our method enables more samples to be analyzed per hour by our method, which saves time and cost when using a large sample size. The precision and accuracy between our method (0.10–7.35% and 98.07–107.45%, respectively) and the previous study (1.83–7.05% and 93.7–103.1%, respectively) were also comparable.

In conclusion, a UPLC-MS/MS methods for determining plasma levels of duloxetine and TA in beagle dogs was developed in the present study. This method exhibited excellent linearity, sensitivity, accuracy, precision, and repeatability, and was capable of determining PK parameters following duloxetine or TA administration. The PK parameters of duloxetine and TA were statically similar between the co-administrations of 2 single drugs and the fix-dose combination formulation.

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