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Determination of *N*-acetyl retigabine in dog plasma by LC/MS/MS following off-line μElution 96-well solid phase extraction

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Abstract

A high throughput off-line μElution 96-well solid phase extraction (SPE) followed by liquid chromatography with tandem mass spectrometry (LC/MS/MS) quantification for the determination of *N*-acetyl retigabine in dog plasma has been developed and validated. The method involves the use of μElution 96-well SPE for the simultaneous extraction of *N*-acetyl retigabine and rapid removal of its *N*-glucuronide metabolite that has shown to be problematic due to its instability using other clean-up methods. The μElution SPE technology eliminates the need for post-extraction solvent evaporation and greatly reduces sample preparation time consequently improving assay efficiency.

Keywords: *N*-Acetyl retigabine; μElution SPE; LC/MS/MS; Dog plasma

1. Introduction

N-acetyl retigabine (*N*-[2-amino-4-(fluoro-benzylamino)-phenyl]-acetamide, **I**, Fig. 1) is an active metabolite of retigabine (*N*-[2-amino-4-(fluoro-benzylamino)-phenyl]carbamic acid ethyl ester, **II**, Fig. 1) that is currently under clinical development for the treatment of epilepsy seizures [1,2]. Retigabine demonstrated unique anticonvulsant activity through its opening effect on potassium channels, which stabilizes hyperexcitable neuronal cell membrane and its positive influences on the synthesis of γ-aminobutyric acid (GABA) [3]. Acetylation of retigabine after oral dosing has been proven to be one of the major metabolic pathways [4]. The product of this metabolic pathway, *N*-acetyl retigabine has also shown anticonvulsant activity in animal models [5]. Accurate determination of *N*-acetyl retigabine in biological matrices is crucial for pharmacokinetic and toxicokinetic evaluation of retigabine and *N*-acetyl retigabine.

Recent in vitro and in vivo metabolite profiling studies of retigabine and *N*-acetyl retigabine have revealed other predominant Phase II metabolites, primary and secondary *N*-glucuronide conjugates of both retigabine [3,6] and *N*-acetyl retigabine. The *N*-glucuronide metabolites were found to be easily converted back to their parent drugs during sample preparation and sample analysis. The bioanalytical results of samples with presence of this kind of liable metabolites may lead to false interpretation of pharmacokinetic data and therefore, delay the drug development process [7]. So far, there is only one method being published for the determination of retigabine and *N*-acetyl retigabine in plasma based on our survey of the literature [8]. Although this on-line LC/MS/MS method was fast and sensitive, the coexisting of *N*-glucuronide conjugates and their parent drugs in the diluted plasma injection solutions were not avoided and therefore the conversion of *N*-glucuronide conjugates back to their parent drugs during the course of on-line analysis was not prevented or stopped.

The aim of this study was to develop a sample preparation procedure along with an LC/MS/MS method that efficiently eliminates the interference from the conversion of *N*-glucuronide conjugates and thus accurately determine *N*-acetyl retigabine in dog plasma to support pharmacokinetic and toxicokinetic studies.

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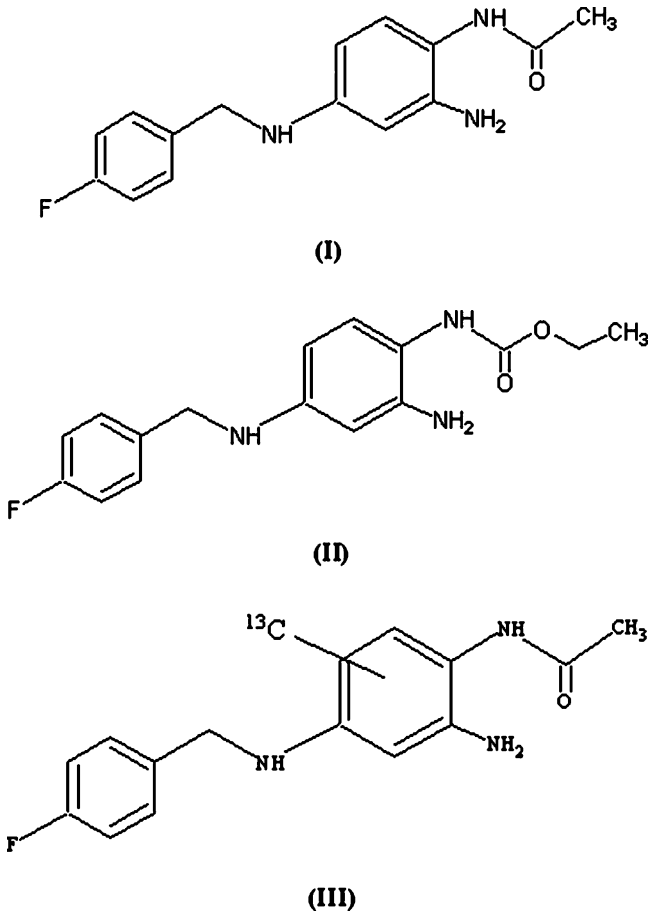

Fig. 1. Chemical structures of *N*-acetyl retigabine (**I**), retigabine (**II**), and [$^{13}C_6$]*N*-acetyl retigabine (**III**).

2. Experimental

2.1. Materials

N-acetyl retigabine (**I**) and [$^{13}C_6$]*N*-acetyl retigabine (**III**) were synthesized by Viatris GmbH & Co. (KG) and Moravek (Brea, CA, USA), respectively. The chemical structures of *N*-acetyl retigabine and [$^{13}C_6$]*N*-acetyl retigabine are shown in Fig. 1. Oasis HLB μElution plates were purchased from Waters Co. (Milford, MA, USA). Control blank beagle plasma (K_3EDTA as anticoagulant) was purchased from Bioreclamation (Hicksville, NY, USA). Ammonium acetate and 2-propanol (IPA) were purchased from Sigma (St. Louis, MO, USA). HPLC-grade water, acetonitrile, methanol and acetic acid (Certified A.C.S PLUS) were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Preparation of standards and quality control samples

Stock solutions of *N*-acetyl retigabine and [$^{13}C_6$]*N*-acetyl retigabine were prepared by dissolving accurately weighed standards in acetonitrile-water (60:40, v/v) to yield a concentration of 0.5 mg/mL for *N*-acetyl retigabine and 0.4 mg/mL for [$^{13}C_6$]*N*-acetyl retigabine, respectively. An internal standard (IS) sub-stock containing 25 μg/mL was prepared by transferring 625 μL of [$^{13}C_6$]*N*-acetyl retigabine stock into a 10 mL volumetric flask and bring to volume with acetonitrile-water (60:40, v/v).

Standard working solutions of *N*-acetyl retigabine at eight concentration levels were prepared by diluting stock solution with acetonitrile-water (60:40, v/v). An IS working solution containing 50 ng/mL of [$^{13}C_6$]*N*-acetyl retigabine was prepared by transferring 50 μL of IS sub-stock into a 50 mL volumetric flask and bring to volume with 4 mM ammonium acetate buffer.

Duplicate calibration standards at eight concentration levels were prepared by spiking 460 μL of control blank plasma with 40 μL of standard working solutions to yield a calibration concentration range of 1 to 1000 ng/mL. The calibration standards were prepared fresh at each analysis. Quality control (QC) samples at four concentration levels (1.0, 3.0, 450 and 850 ng/mL) were prepared by spiking an aliquot of control blank plasma with appropriate volumes of standard working solutions. QC samples were stored at −70 °C until analysis.

2.3. Plasma samples extraction

Study samples and QC samples were thawed at room temperature, mixed thoroughly by vortexing, centrifuged at 3000 rpm (4 °C). For analyte stability concern, calibration standards (freshly prepared), QC and study samples after thawing were placed on ice prior to and during the extraction. All sample dilution and μElution solid phase extraction (SPE) were conducted on ice by placing the 96-well plate and SPE manifold on ice. To a 2.2 mL 96-well plate, 200 μL aliquot of plasma samples were added to the designated wells followed by the addition of 200 μL of IS working solution (except the double blanks to that 200 μL of 4 mM ammonium acetate buffer were added). The plate was capped with a 96-well mat, vortexed for 1 min at mid speed setting and then centrifuged at 3000 rpm, 4 °C for 10 min. Aliquots of 300 μL from each well were transferred using a 12-channel multiple pipettes, to an Oasis HLB μElution SPE 96-well plate pre-conditioned with 200 μL of methanol and equilibrated with 200 μL of 4 mM ammonium acetate buffer. The loaded samples were drawn under vacuum adjusted to an optimized pressure. The wells in the plate were then washed sequentially with 200 μL of 4 mM ammonium acetate:ACN/IPA (40/60), 95:5, v/v (washing solvent A) once to remove matrix interferences and 200 μL of 4 mM ammonium acetate:ACN/IPA (40/60), 80:20, v/v (washing solvent B) twice to completely remove *N*-glucuronide conjugate of *N*-acetyl retigabine. After drying the plate under vacuum for approximate 1 min, the retained analyte and IS in the plate were eluted to a 1.2 mL 96-well plate with 2 × 50 μL of ACN/IPA (40/60, v/v) under vacuum. The final dilution was made by drawing through each well with an aliquot of 100 μL of 4 mM ammonium acetate buffer. The plate was vortexed briefly and 10 μL of diluted samples were injected for LC/MS/MS analysis.

2.4. Chromatographic conditions

The chromatographic separation was performed on a Waters Atlantis dc18 column (20 mm × 4.6 mm, 3 μm). A mobile phase

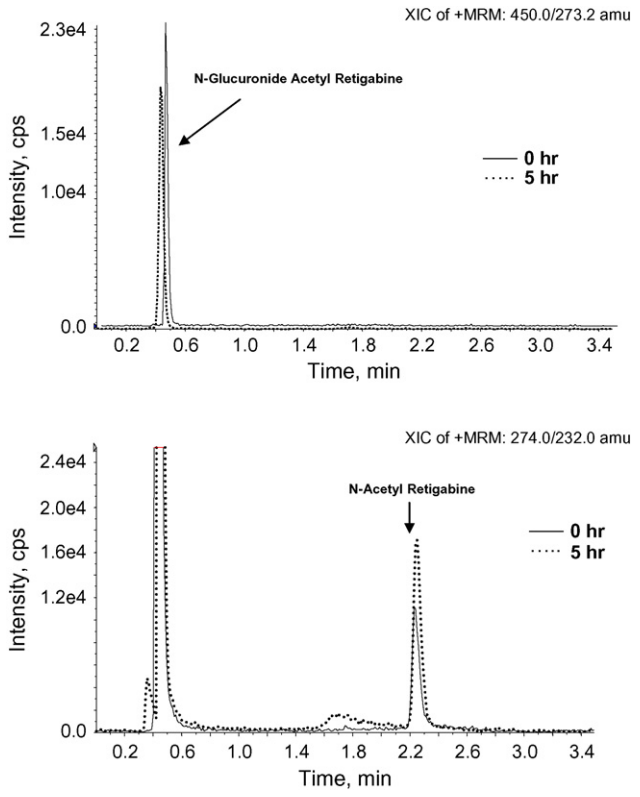

Fig. 2. Conversion of *N*-glucuronide acetyl retigabine to *N*-acetyl retigabine in a post-PPT extract after 5 h storage in autosampler at 4 °C, represented by the changes of extracted ion chromatogram of positive multiple reaction monitoring (XIC of +MRM) of *N*-glucuronide acetyl retigabine (top panel) and *N*-acetyl retigabine (bottom panel). Solid line: 0 h, dash line: 5 h.

gradient program with solvent A (4 mM ammonium acetate) and solvent B (100% ACN) was applied at a flow rate of 1.0 mL/min. The gradient program started with 30% B for 0.25 min followed by a linear increase in B to 90% from 0.25 to 0.5 min and held at 90% for 1.2 min (from 0.5 to 1.7 min). Mobile phase B was then increased to 95% within 0.01 min, held at 95% for another 0.79 min (from 1.71 to 2.5 min) and then reduced linearly to the initial condition (30% B) within 0.1 min. This condition was held until the end of the run. The total run time was 3.5 min.

2.5. APCI-MS/MS conditions

An PE Sciex API 4000 triple quadrupole mass spectrometer, operated in positive atmospheric pressure chemical ionization (APCI) mode, was used for mass detection and analysis. Multiple reaction monitoring (MRM) was used to monitor the precursor → product ion transitions of *m/z* 274 → 232 and 280 → 238 for *N*-acetyl retigabine and [$^{13}C_6$]*N*-acetyl retigabine, respectively (Fig. 2). Dwell time for both transitions was 150 ms. The APCI ion source temperature was at 400 °C. Other optimized MS/MS parameters were: curtain gas flow: 10 psi, collision gas: 6 psi, nebulizer current: 3.0 μA, ion gas 1: 40 psi, ion gas 2: 50 psi, entrance potential: 10 V, collision cell exit potential: 15 V, declustering potential: 91 V for retigabine and 71 V for [$^{13}C_6$]*N*-acetyl retigabine, and collision energy: 25 eV for both compounds.

2.6. Drug administration and plasma sample collection

The toxicokinetics of *N*-acetyl retigabine were evaluated in male and female dogs following daily oral administration. Male and female dogs were given daily oral administration of *N*-acetyl retigabine at a dose of 30, 100, or 600/300/200/100* mg/kg for at least 13 weeks. Dose level for high dose group was decreased from the initial dose of 600 to 300 mg/kg/day beginning on Day 6, to 200 mg/kg/day beginning on Day 12, and to 100 mg/kg/day beginning on Day 32. Plasma samples analyzed were collected from each animal on dosing at pre-dose and at 1, 2, 3, 4, 6, 12, and 24 h post-dose on Week 14.

2.7. Toxicokinetic analysis

The plasma concentration-versus-time data of *N*-acetyl retigabine from individual animal was analyzed at Week 13 and 14 to determine the toxicokinetic parameters using a non-compartmental method in WinNonlin Professional version 4.0 (Pharsight, Mountain View, CA). The maximum concentrations (C_{max}) and times of occurrence for maximum (peak) drug concentration (T_{max}) were the observed values. The area under the plasma concentration-versus-time curve, from 0 to 24 h, ($AUC_{0–24\,h}$) was calculated using the linear trapezoidal-rule option in WinNonlin. Plasma concentrations that were below the limit of quantification (BLQ) were taken as zero for calculations.

3. Results and Discussion

3.1. Sample extraction optimization

The instability of *N*-glucuronide is well known as converting back to their parent drugs at basic and acidic pH's, and at high temperatures [7]. One of the major metabolites of retigabine in human is *N*-acetyl retigabine [5]. Similar to retigabine, *N*-acetyl retigabine containing both primary and secondary amino groups was also found to metabolize to its *N*-glucuronide in dog. Even at neutral pH, the fast conversation of *N*-glucuronide acetyl retigabine to acetyl retigabine was observed using protein precipitation (PPT) without drying and conventional solid phase extraction (data not shown). An on-line LC/MS/MS method [8] provided a fast and sensitive way to determine retigabine and *N*-acetyl retigabine in biologic matrices. However, during the course of on-line analysis *N*-glucuronide conjugates of both retigabine and *N*-acetyl retigabine coexisted with their parent drugs and thus the conversion from the *N*-glucuronide conjugates to their parent drugs were not avoided. A significant increase of *N*-acetyl retigabine along with the decrease of its *N*-glucuronide were detected in a post-PPT extract at neutral pH after 5 h storage in autosampler at 4 °C (Fig. 2). The impossibility of stopping conversion of *N*-glucuronide acetyl retigabine to its parent drug was the drive to develop a method that can quickly remove *N*-glucuronide acetyl retigabine before its conversion and therefore, accurately quantify the parent drug, *N*-acetyl retigabine in dog plasma. Waters 96-well HLB μElution SPE Plate consisting of only 2 mg high capacity sorbent enables the fast complete removal of problematic instable metabolite, *N*-glucuronide acetyl retiga-

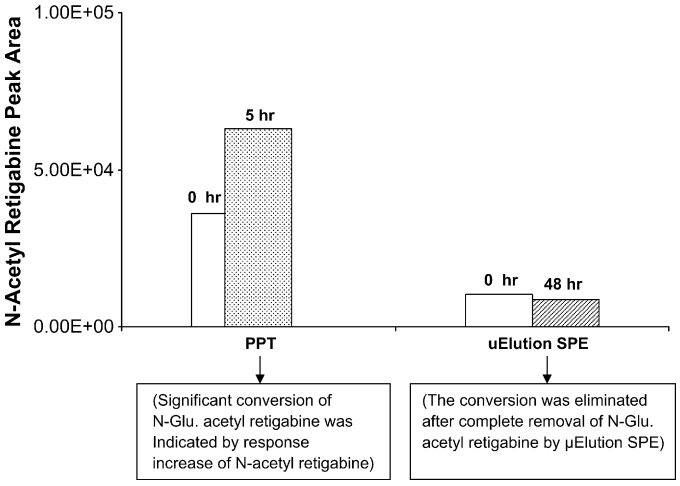

Fig. 3. Comparison of PPT and μElution SPE on *N*-glucuronide acetyl retigabine conversion effect.

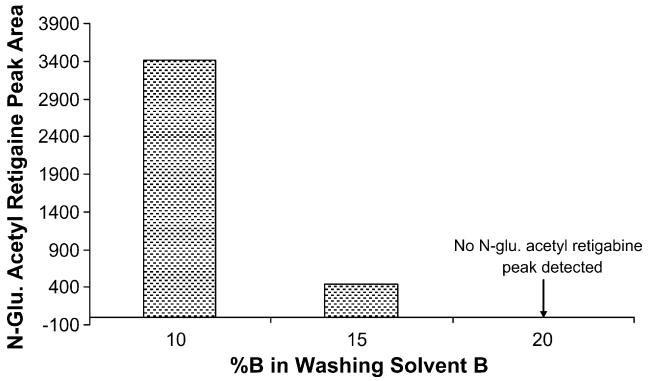

Fig. 4. Effect of percentage of organic (%B) in washing solvent B on removal of *N*-glucuronide acetyl retigabine in μElution SPE. A complete removal of *N*-glucuronide was achieved using using 2 μL × 200μL of 20% ACN/IPA (40/60) in 4 mM ammonium acetate as washing solvent B.

bine before its conversion to the parent drug. The unique design of the μElution SPE Plate allows high loading volume (up to 750 μL), small elution volume (as small as 25 μL) and eliminates the time consuming steps – evaporation and reconstitution [9]. There was no conversion during the μElution SPE and the post-extract was stable for at least 48 h in autosampler at 4 °C (Fig. 3).

To achieve the complete removal of *N*-glucuronide acetyl retigabine while minimize the loss of its parent drug, washing solvent B with different percentage of organic (%B) in buffer (5, 10, 15, 20, 25, 30, 35 and 40%) were evaluated. The optimized condition for washing solvent B was two times washing (2 × 200 μL) with 20% of ACN/IPA (40/60) in 4 mM ammonium acetate buffer (Fig. 4).

The elution solvent condition was investigated and optimized. The 2 × 50 μL elution solvent of ACN/IPA (40/60) was found to give the optimal recovery.

3.2. LC/MS/MS Conditions

The MRM transitions were determined and the corresponding mass spectrometry parameters were optimized by tuning the instrument with the infusion of *N*-acetyl retigabine and IS solutions dissolved in 4 mM ammonium acetate:ACN/IPA (40/60), 50:50, v/v. For stability concern, ammonium acetate buffer at neutral pH (∼6.8 without pH adjustment) was used as mobile phase A and acetonitrile was chosen as mobile phase B. A gradient elution program was developed to obtain the best retentions of *N*-acetyl retigabine and IS with short run time. The relationship between concentration of ammonium acetate and assay sensitivity was studied. The better sensitivity was observed at concentration of 4 mM ammonium acetate buffer. Among several reverse phase columns tested Atlantis dc 18 yielded the best peak symmetry and therefore it was selected. Although both electrospray (ESI) and APCI were applicable to this study, under the same LC condition APCI provided slightly better sensitivity and it was chosen instead of ESI.

3.3. Method validation

3.3.1. Specificity, sensitivity and matrix effect

Specificity of the method was assessed by extracting and analyzing dog plasma double blank from six different lots (three males and three females). As shown in Fig. 5, there was no interference peaks appeared at the retention times of either analyte or internal standard.

Even though a relatively significant loss of *N*-acetyl retigabine was observed during complete removal of its *N*-glucuronide metabolite by μElution SEP, a two and half times better sensitivity than that published [8] with lower limit of quantitation (LLOQ) of 1.0 ng/mL was still achieved. The LLOQ was determined based on a signal-to-noise (S/N) ratio of at least 10. The accuracy and precision at LLOQ determined in

Table 1
Intra-assay and inter-assay precision and accuracy

Assay type	Nominal concentration	*n*	Mean calculated concentration	Precision (% CV)	Accuracy (% of nominal)
Intra-assay	1.00	6	0.879	17.3	87.9
	3.00	6	3.22	13.4	107
	450	6	418	3.30	92.9
	850	6	846	1.18	99.5
Inter-assay	1.00	33	1.05	14.8	105
	3.00	33	2.98	11.2	99.3
	450	33	440	5.86	97.8
	850	33	858	3.32	101

intra-assay ($n = 6$) were 17.3 and 87.9% (Table 1). The representative chromatograms of *N*-acetyl retigabine at LLOQ and at upper limit of the quantitation (ULOQ) are shown in Fig. 5.

The variation due to the use of different source of matrix was defined as relative matrix effect. The relative matrix effect was evaluated by extracting and analyzing triplicate standard samples from each of six different lots of dog plasma. The precision of each set of triplicates ranged from 0.215 to 3.58% and the overall precision was 2.76%, which indicated that there was no matrix effect between different sources of dog plasma.

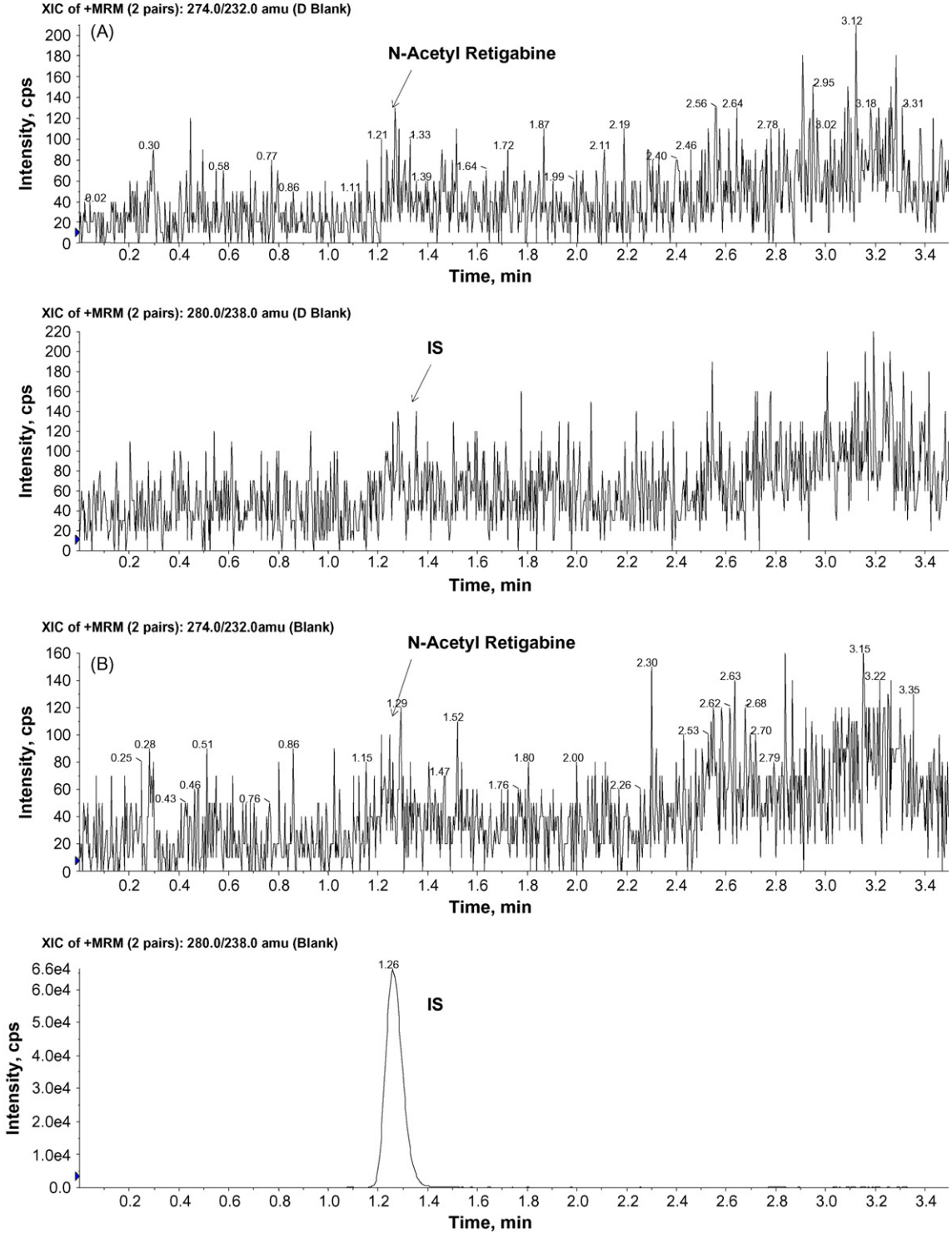

Fig. 5. Representative chromatograms of an extracted dog blank plasma without IS (A), an extracted dog blank plasma with IS (B), an LLOQ plasma standard at 1.00 ng/mL (C) and an ULOQ plasma standard at 1000 ng/mL (D).

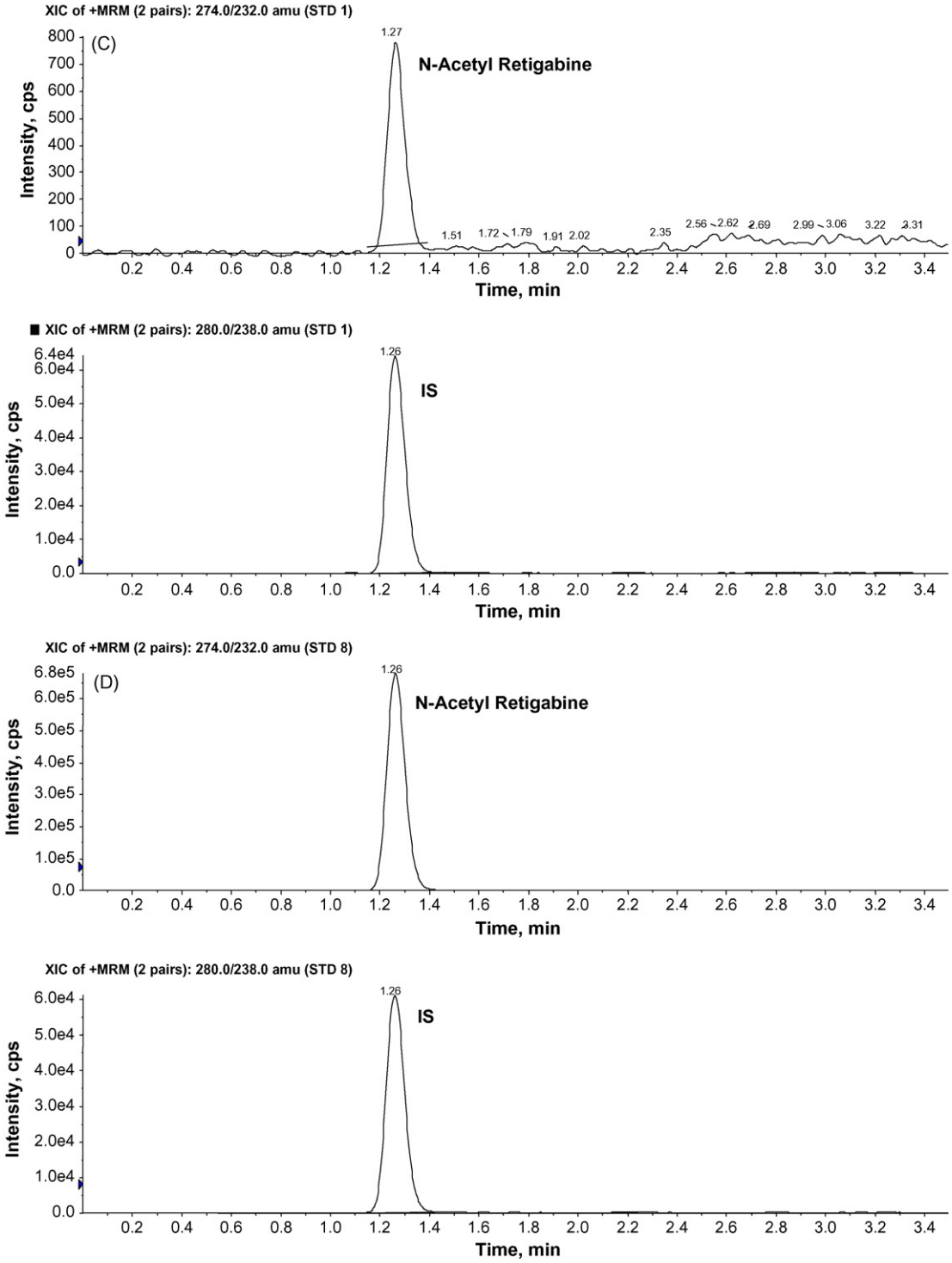

Fig. 5. (*Continued*).

3.3.2. Precision and accuracy

The intra-assay precision and accuracy were evaluated by analyzing within the same run six replicate QC samples at each of four concentration levels. The intra-assay precision ranged from 1.18 to 17.3%, and the accuracy, expressed as percentage of nominal values, ranged from 87.9 to 107% (Table 1). The inter-assay precision determined by analyzing triplicate QC samples at each of four concentration levels for ten sets of runs (including one intra-assay), were between 3.32 and 14.8% and inter-assay accuracy ranged from 97.8 to 105% (Table 1).

3.3.3. Calibration reproducibility

In each of validation sessions, duplicate fresh calibration samples at eight concentration levels were prepared and analyzed as described above. The calibration curves were linear using weighted (1/concentration) least-squares linear regres-

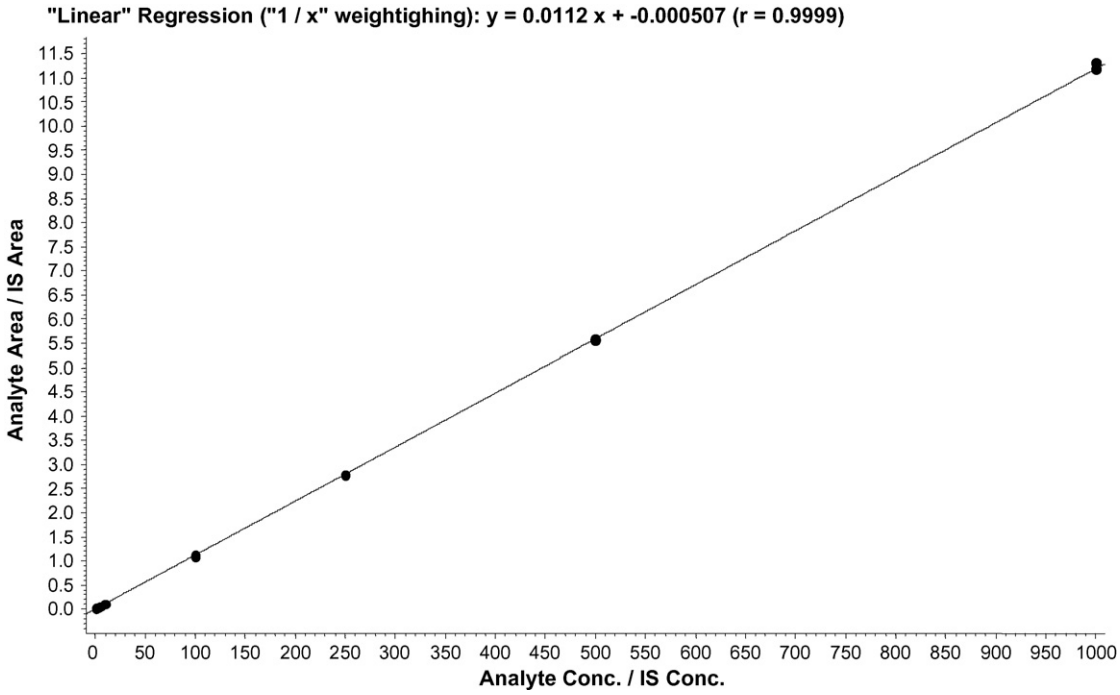

Fig. 6. A representative calibration curve.

sion mode over a concentration range of 1 to 1000 ng/mL, with correlation coefficients (r) equal to or greater than 0.9993. A representative calibration curve is shown in Fig. 6 and the linear regression parameters representing calibration reproducibility are listed in Table 2.

3.3.4. Extraction recovery

The recovery of *N*-acetyl retigabine from dog plasma by μElution SPE was evaluated by comparing peak area ratios (analyte/internal standard) of pre-spiked with that of post-spiked QC samples. The internal standard was spiked after the extraction in both cases. The complete removal of *N*-glucuronide acetyl retigabine was accompanied with the loss of *N*-acetyl retigbine which led a relatively low recovery of 34.8%. Nevertheless the good sensitivity was still achieved with LLOQ of 1.00 ng/mL.

3.3.5. Stability

After successful removal of *N*-glucuronide acetyl retigabine, the post-extract samples were found stable for at least 48 h in

Table 2
Summary of calibration regression results

Assay no.	Intercept (b)	Slope (a)	Correlation coefficient (r)
1	−0.000507	0.0112	0.9999
2	−0.000788	0.0120	0.9999
3	−0.00281	0.0122	0.9999
4	−0.00192	0.0140	0.9998
5	−0.00215	0.0131	0.9999
6	−0.00610	0.0127	0.9998
7	−0.00728	0.0136	0.9993
8	−0.00684	0.0171	0.9994
9	−0.00252	0.0115	0.9997
10	−0.000843	0.0143	0.9997

Linear regression, $y = ax + b$, weighing $1/x$.

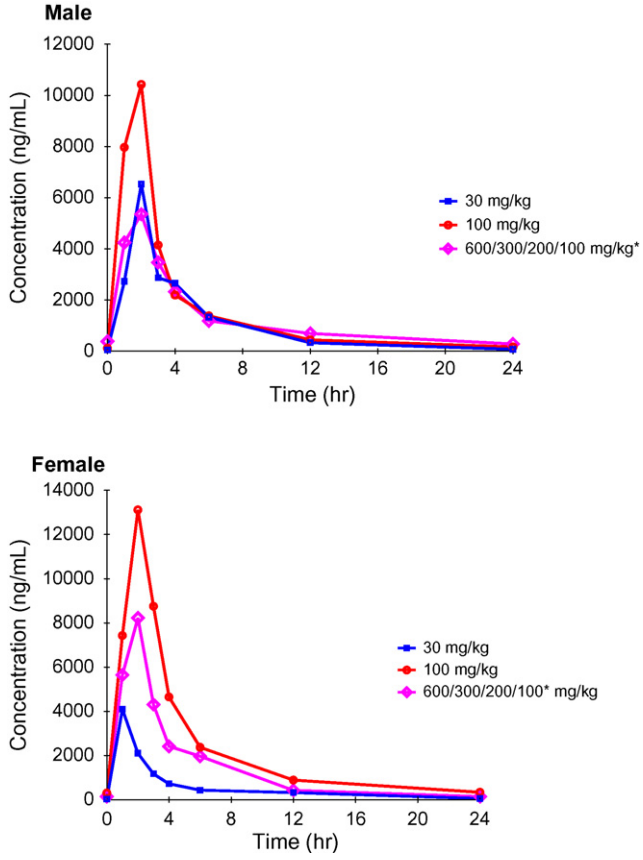

Fig. 7. Plasma concentration–time profiles of *N*-acetyl retigabine in male or female dogs following daily administration of *N*-acetyl retigabine at 30, 100, or 600/300/200/100* mg/kg at Week 14.

Table 3
Mean toxicokinetic parameters (%CV) of *N*-acetyl retigabine following daily oral administration of *N*-acetyl retigabine in dogs at Week 14

Gender	Dose (mg/kg)	*N*	$AUC_{0-24\,h}$ (ng h/mL)	C_{max} (ng/mL)	T_{max}[a] (h)
Male	30	4	24900 (24.0)	7440 (14.9)	2.50 (2.00–4.00)
	100	3	36400 (40.3)	12300 (49.0)	1.33 (1.00–2.00)
	600/300/200/100[b]	4	29500 (52.5)	5570 (104.9)	1.75 (1.00–2.00)
Female	30	3	13500 (37.4)	4100 (49.1)	1.00 (1.00–1.00)
	100	3	55900 (52.5)	13100 (73.6)	2.00 (2.00–2.00)
	600/300/200/100[b]	4	34400 (48.8)	9390 (74.2)	2.75 (1.00–6.00)

N: number of animals.
[a] Mean (range).
[b] Dose level was decreased from the initial dose of 600 to 300 mg/kg/day beginning on Day 6, to 200 mg/kg/day beginning on Day 12, and to 100 mg/kg/day beginning on Day 32.

autosampler at 4 °C with percentage of loss ranged from −5.90 to 3.11% for all QC samples tested at four concentration levels. After three freeze/thaw cycle, *N*-acetyl retigabine in dog plasma demonstrated acceptable stability with percentage loss ranged from −9.95 to −7.82% for low quality control (LQC) and high quality control (HQC) samples. The long-term storage stability (−70 °C) was evaluated by analyzing experimental samples at high and low concentration levels. The results indicated that the dog plasma samples were stable for at least 12 days at −70 °C.

3.4. Toxicokinetic results

The plasma concentration–time profiles of *N*-acetyl retigabine in male and female dogs following daily oral administration of *N*-acetyl retigabine at 30, 100, or 600/300/200/100* mg/kg at Week 14 are illustrated in Fig. 7. The estimated mean toxicokinetic parameters of *N*-acetyl retigabine are summarized in Table 3.

Following daily oral dosing of *N*-acetyl retigabine, the time to reach the maximum plasma concentration (T_{max}) of *N*-acetyl retigabine was variable, ranging 1.0 to 4.0 h and 1.0 to 6.0 h in male and female dogs, respective. $AUC_{0-24\,h}$ values of *N*-acetyl retigabine increased with doses ranging from 30 to 100 mg/kg in both male and female dogs at Week 14. However, there was a marginal decrease in $AUC_{0-24\,h}$ values for male and female dogs from dose 100 to 600/300/200/100* mg/kg at Week 14. There were no significant differences in $AUC_{0-24\,h}$ or C_{max} between the male and female dogs, indicating an absence of gender effect.

4. Conclusion

A rapid, sensitive and selective μElution 96-well solid phase extraction (SPE) liquid chromatography/tandem mass spectrometry (LC/MS/MS) method has been developed and validated for the determination of *N*-acetyl retigabine in dog plasma. The SPE method was efficient for the simultaneous extraction of *N*-acetyl retigabine and rapid removal of its *N*-glucoronide metabolite from dog plasma. The removal of the *N*-glucoronide metabolite of *N*-acetyl retigabine by SPE allowed the accurate determination of *N*-acetyl retigabine without the drawback of conversion of metabolite back to parent during sample extraction and analysis. The method was linear ($r > 0.9993$) over the concentration range of 1.00–1000 ng/mL. The intra- and inter-day assay accuracy (% of nominal) and precision (%CV) were, respectively, within ±15% (±20% at the LLOQ) and ≤15% (≤20% at the LLOQ). No matrix interference at the retention time of *N*-acetyl retigabine and lot-to-lot variation were observed when the method was tested using six different lots of dog plasma. The validated method was successfully utilized to accurately analyze 263 experimental samples for a toxicokinetic study.

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