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Determination of LAG078, a lipid-lowering compound, in dog plasma using liquid chromatography–tandem mass spectrometry: application in a toxicokinetic study

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Abstract

A high throughput method was developed and validated for the quantitative determination of LAG078, a lipid-lowering compound, in dog plasma obtained during toxicokinetic studies. The method was based on reverse phase liquid chromatographic separation of the analyte from plasma extract followed by turbo-ionspray (TIS) in the negative ion mode and tandem mass spectrometry in the multiple reaction monitoring (MRM) mode. Extraction was performed using a combination of protein precipitation and liquid–liquid extraction in the 96-well plate format to increase the throughput of the method. Optimized chromatographic separation under basic condition (pH $\sim$ 10) in a short polymer based column (50 mm $\times$ 2.0 mm i.d.) coupled with MRM mode of detection yielded clean chromatograms with minimal signal suppression. The standard curve was linear ($r = 0.997$) within the concentration range of 0.05 (lower limit of quantification; LLOQ) to 50 ng/ml using only 0.1 ml of dog plasma. The accuracy of the method varied from 95 to 100% with a precision (CV) of 3.04–10.8% over the concentration range. The method was simple, rapid, and robust.
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1. Introduction

Hyperlipidemia is a major risk factor for atherosclerosis and coronary artery disease. The therapeutic standards for this indication are the statin drugs, which inhibit the HMG CoA reductase enzyme. Some of the commonly used statins are atorvastatin, fluvastatin, simvastatin, prevastatin, and lovastatin. If one defines the signs and symptoms of hyperlipidemia as the elevated clinical laboratory values for total cholesterol, low-density lipoprotein (LDL), and triglycerides. Then the HMG CoA reductase inhibitors provide adequate response in roughly 70–90% of the general hyperlipidemic patients. Despite the potential of these agents to lower LDL, retard progression of coronary atherosclerosis, and reduce the incidence of cardiovascular events, these drugs do not prevent myocardial

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infarctions in a substantial percentage of patients. It has been reported that LDL elevation is only one of the several lipid abnormalities associated with the risk of having a coronary event, including elevated levels of lipoprotein Lp(a), triglyceride-rich lipoproteins, and the magnitude of postprandial lipemia [1]. The well known Framingham study showed that although elevated blood cholesterol reflects a high risk of heart disease, normal blood cholesterol does not necessarily reflect a low risk [1].

A new therapeutic approach for the treatment hyperlipidemia emerged from the important role thyroid hormones (L-T3, L-T4) play on lipid metabolism [2,3]. It has been observed that hypothyroidism in patients results in high plasma levels of cholesterol and low-density lipoprotein leading to increased risk of atherosclerosis [4,5]. On the other hand, hyperthyroid patients have decreased levels of LDL. The hypocholesterolemic effect of thyroid hormones has been well documented in human and laboratory animals, in which it produces an upregulation of LDL receptors and a marked lowering of LDL-cholesterol [2,3]. However, the excessive myocardial activities associated with thyroid hormones and other synthetic thyromimetic agents have limited their use as hypolipidemics [6,7]. The myocardial activities represent both direct effects on the myocardium and indirect effects associated with the elevation in basal metabolic rate, which, by enhancing peripheral tissue oxygen demand, increase the cardiac workload [8]. The high-affinity, low-capacity nuclear receptors for thyroid hormones are believed to be responsible for the initiation of virtually all of the well documented effects of thyroid hormones. Thus, if the access of a thyromimetic was largely limited to the liver nuclei (the site of its hyperlipidemic effects) and access to the nuclei of cardiac and other tissues was reduced or eliminated, a cardiac-sparing hypolipidemic agent should result. Therefore, for a thyromimetic to be clinically useful as a hypolipidemic agent, it should be devoid of all cardiac effects. Based on unpublished preclinical data, this class of compounds is expected to exhibit at least comparable lipid-lowering effects to the HMG CoA reductase inhibitors with respect to total cholesterol and LDL-cholesterol. In addition, they would be expected to decrease Lp(a), a lipoprotein that is also an independent risk factor for cardiovascular disease, and produce beneficial effects on postprandial lipemia, which has been associated with increased risk for coronary artery disease.

LAG078 is a thyromimetic lipid modulator that showed good lipid-lowering activity in the normocholesterolemic dog model while demonstrating no adverse cardiac side effects in the rat cardiovascular safety model [unpublished data]. This report describes a high-throughput and sensitive (LLOQ = 50 pg/ml) LC–MS–MS method for the quantitative determination of LAG078 in dog plasma to support toxicokinetic studies.

2. Experimental

2.1. Chemicals

All the solvents and chemicals were of HPLC or analytical grade and used without further purification. Methanol, acetonitrile, ethyl acetate and ammonium hydroxide were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ammonium acetate was purchased from Fluka (Milwaukee, WI, USA). Water was purified using Milli-Q system from Millipore Corporation (Bedford, MA, USA). Blank dog (beagle) plasma was purchased from Bioreclamation, Inc. (Hicksville, NY, USA). LAG078 ($C_{22}H_{18}FNO_7S$, average MW = 459.5 and monoisotopic mass = 459.1) reference standard and LAB638 ($C_{23}H_{20}FNO_6$, average MW = 425.4 and monoisotopic mass = 425.1) were obtained from Novartis Pharmaceuticals Corporation (E. Hanover, NJ, USA). LAG078 was obtained as free acid monohydrate having an average molecular weight of 477.5 (free acid/mono-hydrate ratio = 0.96). Chemical structures of these compounds are shown in Fig. 1.

2.2. Standard and QC solutions

A stock solution (1.0 mg/ml) of LAG078 was prepared by dissolving 4.69 mg of LAG078 monohydrate (free acid/mono-hydrate ratio of 0.96) in 4.5 ml methanol. Working solutions of the reference standard were prepared at concentrations 0.2, 0.4, 2.0, 4.0, 20, 80, and 200 ng/ml by serial dilution of the stock solution in methanol:water (50:50; v/v). A 25 μl aliquot of each of the working solutions was spiked into 0.1 ml of blank dog plasma to prepare the calibration

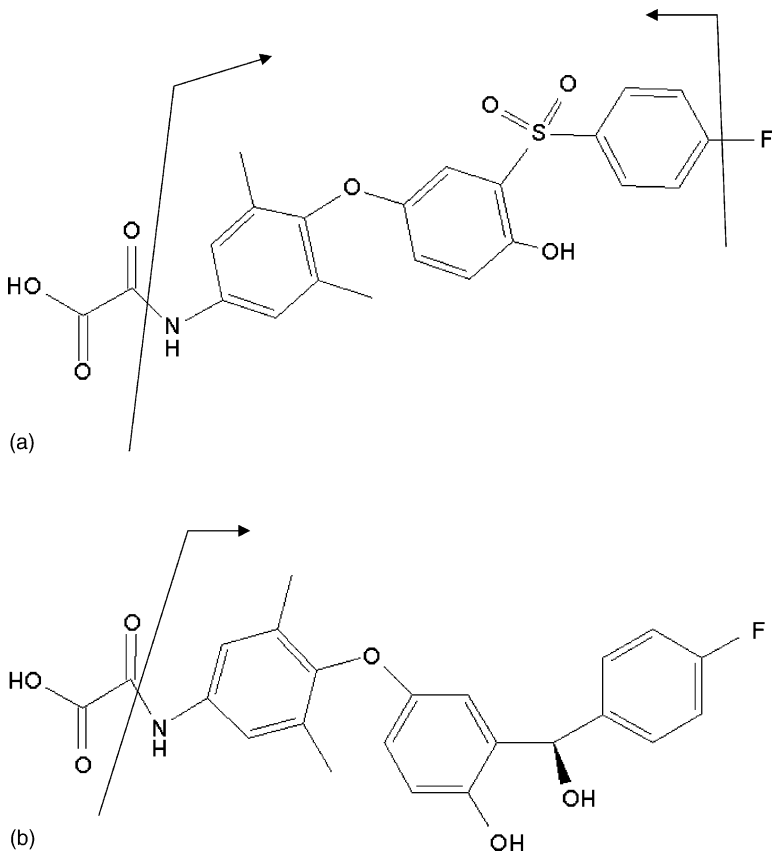

Fig. 1. Structures of (a) LAG078 and (b) the internal standard LAB638. The arrow refers to the resulting bond cleavages during MS–MS to produce the most abundant product ion (*m/z* 366 for LAG078 and *m/z* 352 for the internal standard).

standards at plasma concentrations of 0.05, 0.1, 0.5, 1.0, 5.0, 20, and 50 ng/ml respectively. QC samples of LAG078 were prepared by separately weighing the solid compound and dissolving in solvents as described for the calibration standards. QC samples were prepared in blank dog (beagle) plasma (mentioned in Section 2.1) at concentrations of 0.05, 0.15, 0.75, 2.5, 15, and 40 ng/ml and stored at −20 °C pending analysis. A stock solution of the internal standard (IS) LAB638 was prepared in methanol at 0.052 mg/ml. A working solution (31.2 ng/ml) of the IS was prepared by diluting the stock solution in methanol:water (50:50; v/v). A 25 μl aliquot of the working solution was spiked into each dog plasma sample (0.1 ml) during the analysis to yield a final concentration of 7.8 ng/ml.

2.3. Liquid chromatography

The chromatographic separations were performed using an Asahipak ODP C_{18} (50 mm × 2.0 mm i.d., 5 μm particle size) column (Thermo Hypersil Keystone, Bellefonte, PA, USA). The stationary phase of the column was based on polyvinyl alcohol polymer and was stable from pH 3 to 13. The column was operated at ambient temperature. The LC system consisted of two Shimadzu LC-10ADvp pumps and a SCL-10Avp controller (Shimadzu, Columbia, MD, USA). An on-line solvent degasser (Metachem, Torrance, CA, USA) was also utilized with the LC system. The autosampler was a HTS PAL (Leap Technologies, Carrboro, NC, USA). Table 1 summarizes the chromatographic separation conditions

Table 1
Chromatographic gradient used for the determination of LAG078 in dog plasma

Time (min)	Mobile phase A (%)	Mobil phase B (%)	Flow rate (ml/min)
0.0	80	20	0.08
1.0	80	20	0.08
2.5	5	95	0.08
3.5	5	95	0.08
4.5	80	20	0.08
5.0	80	20	0.08

performed using a gradient of solvent A (0.04% ammonium hydroxide and 10 mM ammonium acetate in water; pH ∼ 10) and solvent B (90% acetonitrile/10% methanol; v/v). The injection volume was 20 μl and the column pressure was approximately 800 psi. The auto injector syringe, and the injector valve were washed sequentially three times with wash-1 acetonitrile:methanol:ammonium hydroxide (80:19:1; v/v/v) and wash-2 (methanol:water (50:50; v/v)).

2.4. Mass spectrometry

All mass spectrometric data were collected using a Sciex API 3000 triple quadrupole mass spectrometer (PE-Sciex, Toronto, Canada) equipped with a turbo-ionspray (TIS) interface. The experiments were performed in negative ionization mode. The nebulizer temperature was 400 °C and the ion spray voltage was −4.2 kV. Nitrogen gas was used as both the nebulizer gas and the curtain gas at a setting 8 to assist liquid nebulization and desolvation. Scanning was performed in multiple reaction monitoring (MRM) mode. The collision energy was 25 eV for both LAG078 and the IS. Nitrogen was used as the collision gas at setting 8. The electron multiplier voltage was maintained at 2.6 kV. The MS–MS product ion transitions of *m/z* 458 (precursor ion) → 366 (dwell time = 1 s) and *m/z* 424 (precursor ion) → 352 (dwell time = 1 s) were used for LAG078 and the internal standard (LAB638), respectively. Figs. 2 and 3 depict representative MS–MS spectra obtained for LAG078 and the IS.

2.5. Assay procedure

Sample extraction and analysis were performed in 96-well format. The working solutions (25 μl) of calibration standard were added to designated wells in a 96-well 2 ml polypropylene block (Microliter Analytical Supplies Inc., Suwanee, GA, USA). A 25 μl aliquot of methanol:water (50:50; v/v) was added to all the other wells. Blank dog plasma (0.1 ml) was added to each well containing the calibration standards and blanks. The QC samples were thawed to room temperature. A 0.1 ml aliquot of each QC sample was added to a specific well. Working solution (25 μl) of the internal standard was added to each well except the wells designated for blanks where a 25 μl aliquot of methanol:water (50:50; v/v) was added. The content of the wells was mixed by gently shaking the 96-well block.

The next step in the assay procedure involved protein precipitation of the plasma samples followed by liquid–liquid extraction in the same 96-well plate. Protein precipitation was performed by adding a 0.2 ml aliquot of acetonitrile to each well. The plate was vortexed for 5 min. Then a 0.5 ml aliquot of ethyl acetate was added to each well for liquid–liquid extraction. The plate was then vortexed for 10 min and then centrifuged at approximately 2000 × *g* for 10 min. The organic layer from the top was transferred to a clean 96-position square well polypropylene block. All the pipetting steps during protein precipitation and liquid-liquid extraction were carried out on a semi-automated Tomtec Quadra 96 Model 320 workstation (Tomtec, Hamden, CT, USA). The organic extract in the square well block was evaporated using the flow of dry nitrogen gas at 45 °C for 25 min in a 96-channel solvent evaporator (TurboVap 96 Concentration Workstation, Zymark Corporation, Hopkinton, MA, USA). The samples were reconstituted by adding a 75 μl aliquot of methanol:water (50:50; v/v) to each well and vortexing the plate for 10 min. The plate was then centrifuged at 1800 × *g* for 10 min to settle any particulate in the extract. The extract (20 μl) was injected directly to the LC column for analysis.

2.6. Data processing, calibration curve and quantification of samples

Data acquisition and processing were performed using Analyst software, version 1.1 (Sciex Corporation, Toronto, Canada). Statistical analysis was performed using Microsoft Excel, version 6.0 (Microsoft Corporation, Redmond, WA, USA). The calibrators

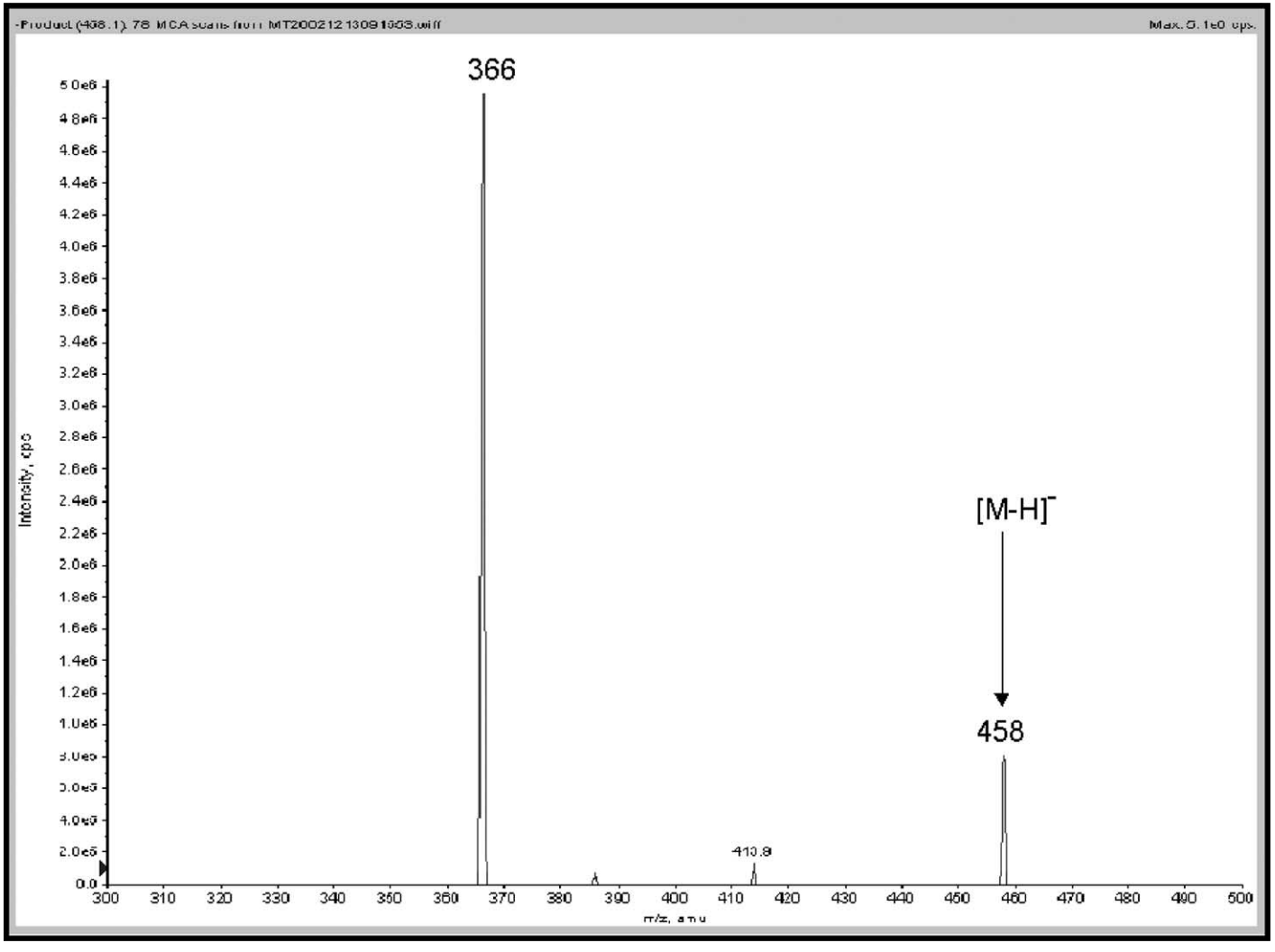

Fig. 2. Representative full scan TIS–MS–MS spectrum of LAG078 (collision energy −25 eV and collision gas pressure at setting 8) showing the product ion at *m*/*z* 366 and the precursor ion at *m*/*z* 458. Mass to charge ratio (*m*/*z*) is shown on the horizontal axis and the ion intensity is shown in the vertical axis.

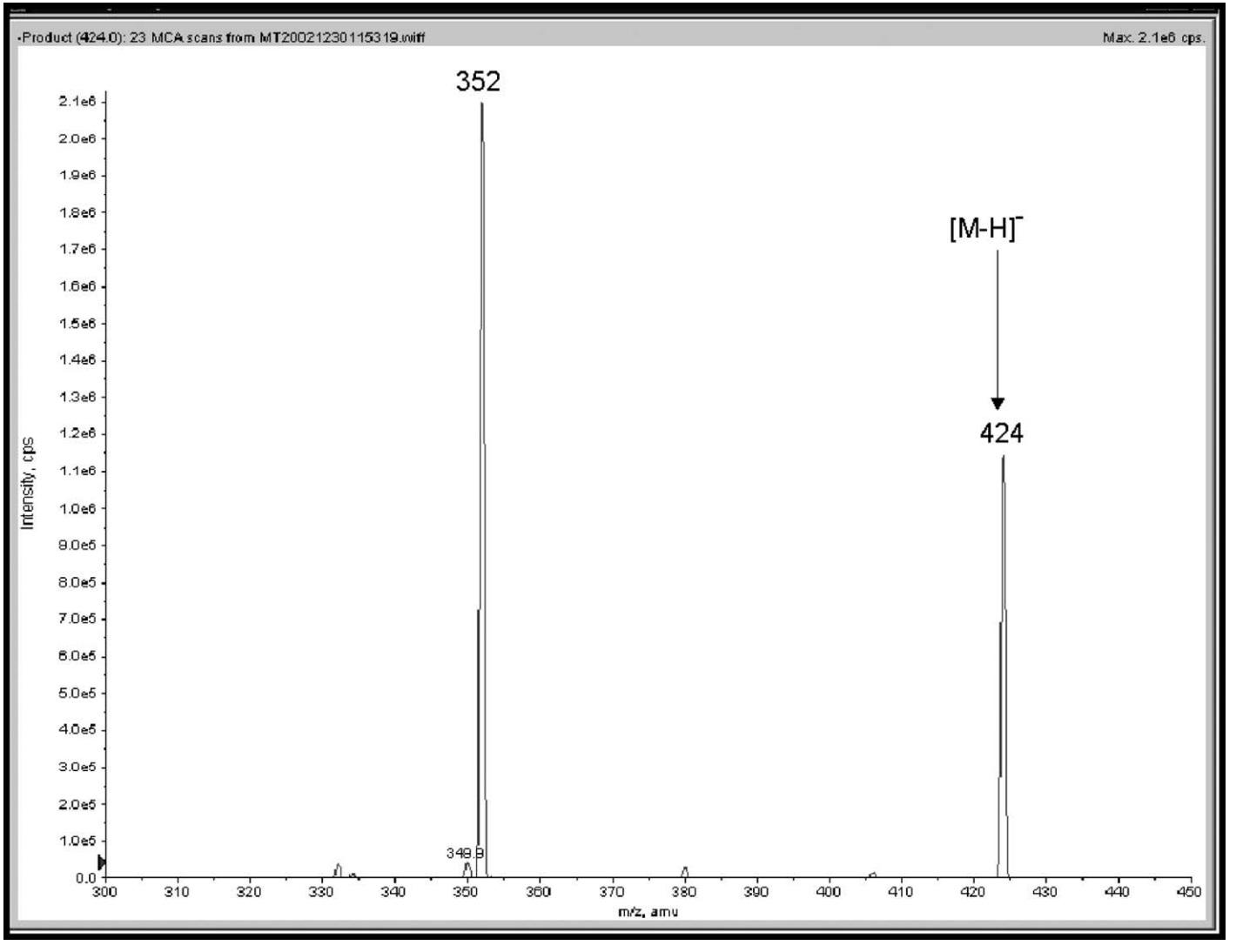

Fig. 3. Representative full scan TIS–MS–MS spectrum of the internal standard LAB638 (collision energy −25 eV and collision gas pressure at setting 8) showing the product ion at *m*/*z* 352 and the precursor ion at *m*/*z* 424. Mass to charge ratio (*m*/*z*) is shown on the horizontal axis and the ion intensity is shown in the vertical axis.

Table 2
Summary of calibration curve obtained for the analysis of LAG078 in dog plasma (calibration parameters a and b of the calibration function $y = ax + b$ and correlation coefficient r on each day of the validation)

Analysis day	Nominal concentration (ng/ml)							Slope (a)	y-intercept (b)	Correlation coefficient (r)
	0.05	0.1	0.5	1.0	5.0	20	50			
Back-calculated concentration (ng/ml)										
Day 1	0.051	0.0922	0.489	1.04	4.42	20.2	51.5	0.109	−0.00122	0.998
	0.052	0.0951	0.515	0.992	5.25	21.4	43.0			
Day 2	0.052	0.101	0.500	1.00	4.99	19.6	51.0	0.111	0.00017	0.999
	0.0461	0.105	0.525	0.988	4.88	19.0	50.5			
Day 3	0.0525	0.096	0.431	1.10	4.43	20.4	54.5	0.109	0.00565	0.995
	0.0424	0.0937	0.453	0.914	5.45	21.0	54.5			
Mean	0.0493	0.0972	0.486	1.01	4.90	20.3	50.8	0.110	0.00153	0.997
±S.D.	0.00414	0.00487	0.0365	0.0617	0.421	0.882	4.22	0.00115	0.00363	0.00208
CV (%)	8.40	5.01	7.51	6.11	8.59	4.34	8.31	1.05	242	0.209
n	6	6	6	6	6	6	6	3	3	3
Accuracy (%)	98.6	97.2	97.2	101	98.0	102	102	–	–	–

were processed in duplicate. Calibration curves ($y = ax + b$), represented by the plots of the peak area ratios (y) of LAG078 to internal standard versus the concentrations (x) of LAG078 in the calibration samples, were generated using weighted ($1/x^2$) linear least-squares regression. The LAG078 concentrations were expressed in terms of the free acid. Representative standard calibration curve parameters for LAG078 are shown in Table 2. Concentrations in the QC and stability samples were calculated from the resulting peak area ratios and the regression equation of the calibration curve.

3. Results and discussion

3.1. Chromatography and tandem mass spectrometry

The analytical approach used in this method satisfied the need for a high throughput assay for LAG078 to support preclinical studies. The extraction procedure was optimized to minimize the matrix interference. It was observed during the method development that, LAG078 was retained in the injector after every injection of neat LAG078 solution having concentration higher than 5 ng/ml. The LAG078 peak from the injector carryover was observed in the subsequent solvent blank. The problem was affecting the specificity of the method by adding LAG078 contamination in the low concentration (≤5 ng/ml) samples. The carryover problem was eliminated by adding ammonium hydroxide solution in the injector wash (Section 2.3). The injector wash procedure mentioned in Section 2.3 eliminated the carryover of LAG078 in the injector to the extent that no LAG078 peak was observed in the solvent wash injected following the injection of the 50 ng/ml standard (upper limit of quantification). There was no carryover problem with the IS.

It was also observed during the method development that the signal intensity of LAG078 increased significantly upon post column addition of 0.04% aqueous ammonium hydroxide solution (pH ∼ 10). This signal enhancement could be from the pK values of LAG078. LAG078 has two pK values ($pK_1 = 2.41$ and $pK_2 = 7.74$) due to the presence of both acidic and basic functional groups in the molecule (Fig. 1(a)). Post column addition of ammonium hydroxide solution with pH ∼ 10 probably enhanced the formation of anion from the –COOH group in the molecule. The method was optimized using alkaline mobile phase during LC separation to increase signal intensity of the LAG078 anion in the turbo-ionspray source of the mass spectrometer. The basic mobile phase also probably helped to minimize the carryover problem.

Most silica-based LC columns have an upper pH limit of 8 for their operation. The column used in

this method was a polyvinyl alcohol-based rigid polymer (stable at a pH 13) that was compatible with the basic mobile phase. Smaller column dimension (50 mm × 2.0 mm i.d.) in conjunction with the gradient used in the method (Table 1) resulted in early elution of polar matrix interferences rendering minimal ion suppression typically observed during electrospray ionization [9–13]. The LC–MS–MS of LAG078 exhibited a good sensitivity with linear response within the concentration range of 0.05–50 ng/ml in the negative ion mode using a turbo-ionspray (TIS) interphase. Both specificity and sensitivity of the method was also maximized by using negative mode of ionization in conjunction with MS–MS in the MRM mode. The $[M–H]^-$ ion in the negative ionization mode was intense due to the presence of –COOH group in the molecule that spontaneously formed $–COO^-$ ion under basic condition. A representative MS–MS product spectrum of ion *m/z* 458 $[M–H]^-$ from LAG078 is shown in Fig. 2. The product ion *m/z* 366 $[(M–H)^–C_2O_3HF]$ with highest intensity was selected for MRM scan to achieve good sensitivity. For the IS the MS–MS product ion with *m/z* 352 was formed from the precursor ion $[M–H]^-$ by the fragmentation process $[(M–H)^–C_2O_3]$ and is shown in Fig. 3.

3.2. Calibration curves

The calibration curve parameters from 3 days of validation are summarized in Table 2. The mean correlation coefficient was 0.997. The fit of the calibration standards to each curve (Table 2) was assessed from the accuracy (%): 100 × (back-calculated concentration from the regression line equation)/(nominal concentration). The mean accuracies were within 97.2–102% from theoretical value and indicated a good fit of the regression model over the concentration range 0.05–50 ng/ml of the calibration curve. The coefficient of variation (CV) did not exceed 8.59%.

3.3. Accuracy and precision of the quality control (QC) samples

Both intra- and inter-day accuracy and precision of the method were evaluated from six replicates of QC samples of known concentrations. These QC

Table 3
Accuracy and precision of QC samples for LAG078 in dog plasma

Analysis day	Nominal concentration (ng/ml)					
	0.05	0.15	0.75	2.5	15	40
Day 1 ($n = 6$)						
Intra-day mean	0.0545	0.148	0.705	2.35	14.3	37.4
±S.D.	0.00337	0.0108	0.0200	0.0579	0.304	0.730
CV (%)	6.18	7.30	2.84	2.46	2.13	1.95
Mean accuracy %	109	98.7	94.0	94.0	95.3	93.5
Day 2 ($n = 6$)						
Intra-day mean	0.0496	0.147	0.709	2.34	14.2	36.6
±S.D.	0.00501	0.00469	0.0113	0.0324	0.206	0.331
CV (%)	10.1	3.19	1.59	1.38	1.45	0.904
Mean accuracy %	99.2	98.0	94.5	93.6	94.7	91.5
Day 3 ($n = 6$)						
Intra-day mean	0.0464	0.133	0.765	2.63	14.8	40.2
±S.D.	0.00363	0.00819	0.0495	0.137	0.406	1.96
CV (%)	7.82	6.16	6.47	5.21	2.74	4.88
Mean accuracy %	92.8	88.7	102	105	98.7	101
Inter-day mean ($n = 18$)	0.0501	0.143	0.726	2.44	14.4	38.0
±S.D.	0.00542	0.0110	0.0431	0.164	0.438	2.03
CV (%)	10.8	7.69	5.94	6.72	3.04	5.34
Mean accuracy %	100	95.3	96.8	97.6	96.0	95.0

samples were prepared at six different concentrations spreaded along the calibration curve range. The lowest QC sample of 0.05 ng/ml (Table 3) was used to validate the lowest limit of calibration (LLOQ) of the method. The experiments were repeated on three different validation days and the data are shown in Table 3. Accuracy was determined by calculating the mean recovery for the observed concentrations as percent of the nominal concentrations in QC samples. Precision was assessed from the coefficient of variation (CV) of the mean recoveries. As shown in Table 3, the intra-day mean accuracies varied from 88.7 to 109% over the 0.05–40 ng/ml concentration range of LAG078; the corresponding precision ($n = 6$) varied from 1.38 to 10.1%. The inter-day mean recoveries varied from 95.0 to 100% and the corresponding precision ($n = 18$) varied from 3.04 to 10.8%.

3.4. Lower limit of quantitation

The lower limit of quantitation, defined as the lowest concentration on the standard curve that could be measured with acceptable accuracy and precision, was 0.05 ng/ml using 0.1 ml of dog plasma. The accuracy ($n = 18$) of determination at LLOQ was

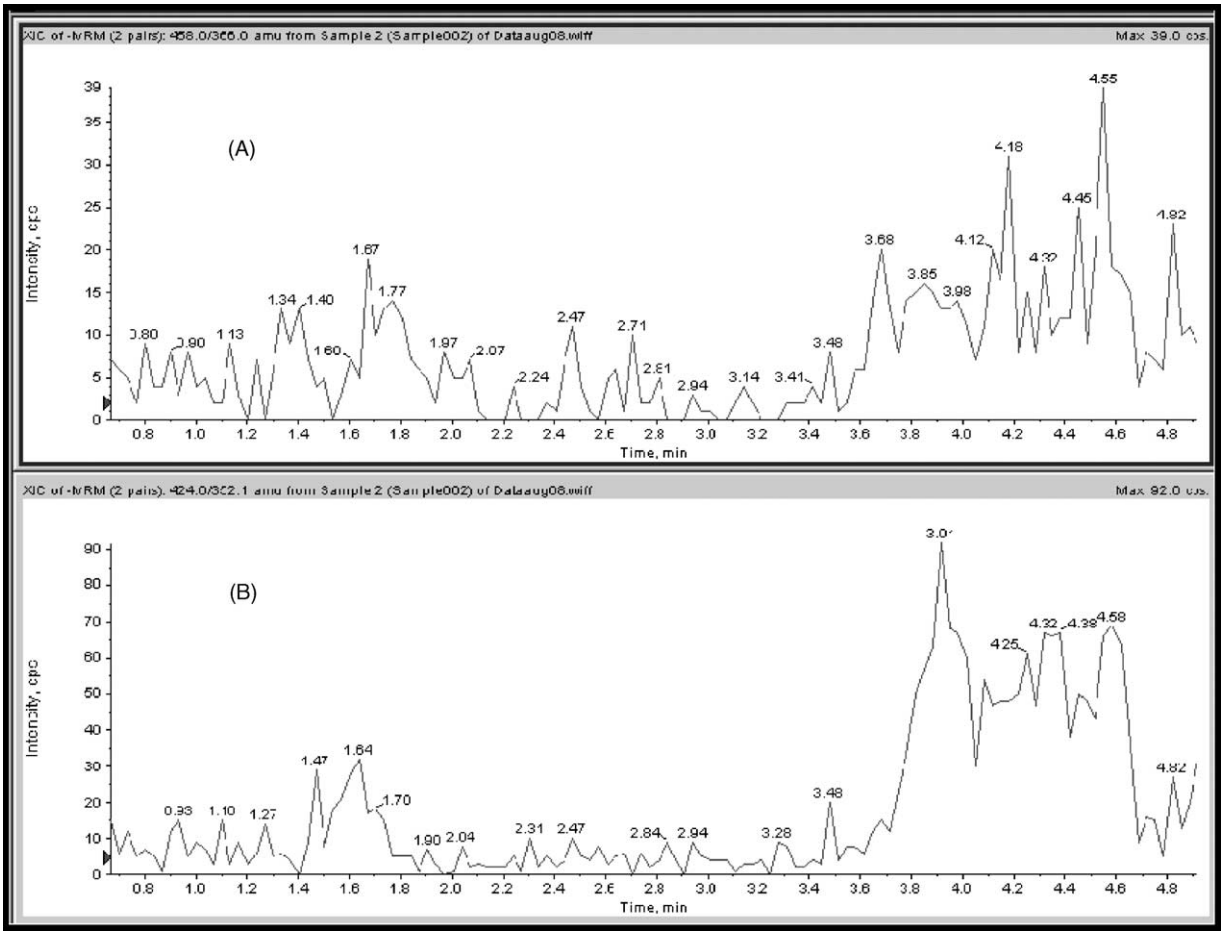

Fig. 4. LC–TIS–MS–MS ion-chromatograms in blank dog plasma. Panel A is the ion chromatogram for LAG078. Panel B is the ion chromatogram for the internal standard. Retention time (min) is shown on the horizontal axis and the peak intensity is shown in the vertical axis.

100% with a precision of 10.8% (inter-day mean; Table 3).

3.5. Specificity

The instrument was operated in the MRM mode to maximize specificity and sensitivity. The fragmentation reactions of *m/z* 458 → 366 and *m/z* 424 → 352 were monitored for LAG078 and the internal standard, respectively. Representative chromatograms of extracts of blank (free of LAG078) dog plasma and of the same plasma spiked with the internal standard are shown in Figs. 4 and 5 respectively. High specificity of the method was shown by the absence of any peak above the noise level in the blank dog plasma sample (Fig. 4). Peak assignment was established from samples spiked individually with each compound and treated as described in the sample preparation procedure. The retention times for LAG078 and the internal standard were the same and equal to approximately 3.85 min. Lack of "cross-talk" in the presence of the internal standard was shown by the absence of any peak in the MRM channel of LAG078 (upper trace of Fig. 5) at the appropriate retention time (~3.85 min). Ion chromatograms of LAG078 at the LLOQ (0.05 ng/ml) and the internal standard

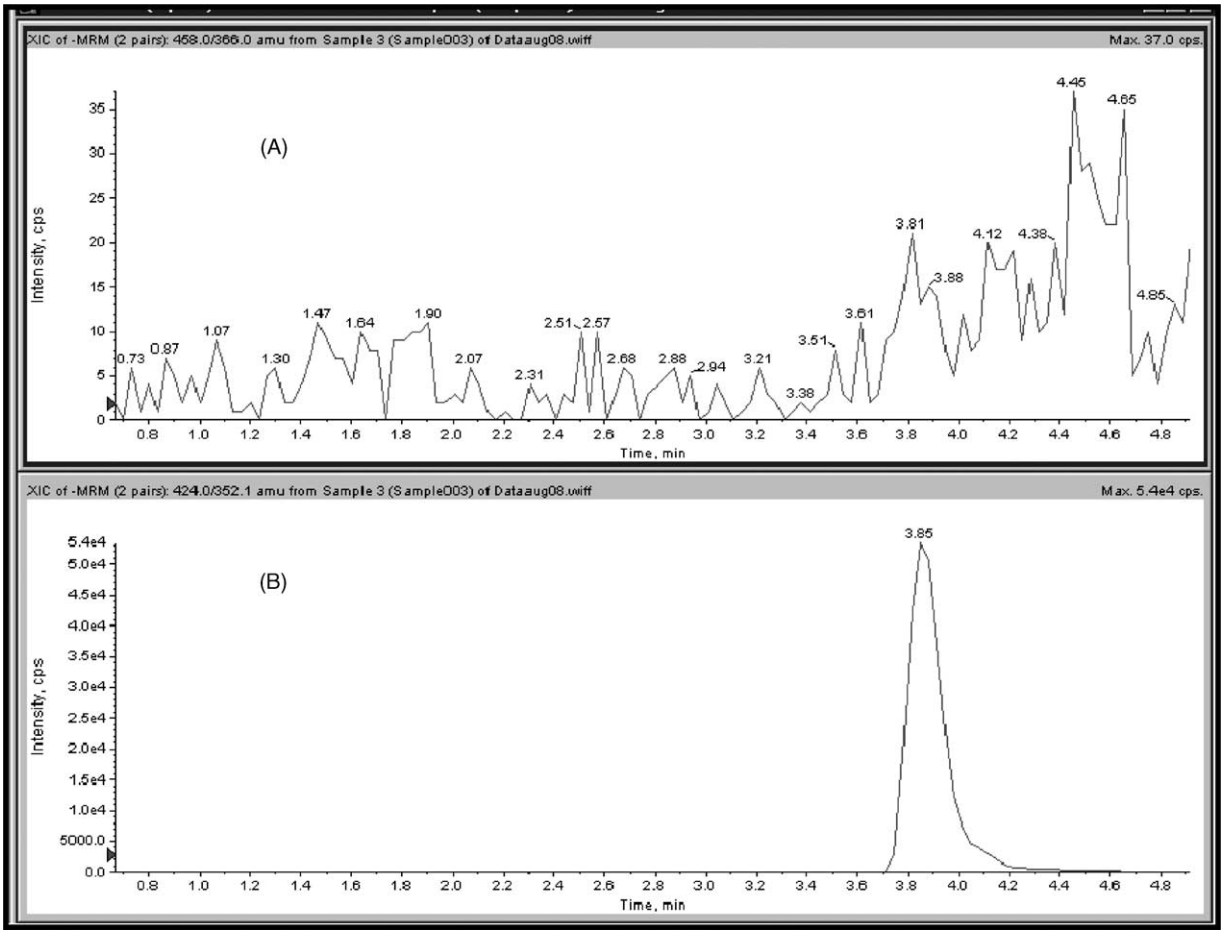

Fig. 5. LC–TIS–MS–MS ion-chromatograms in blank dog plasma spiked with the internal standard (7.8 ng/ml). Panel A is the ion chromatogram for LAG078. Panel B is the ion chromatogram for the internal standard. Retention time (min) is shown on the horizontal axis and the peak intensity is shown in the vertical axis.

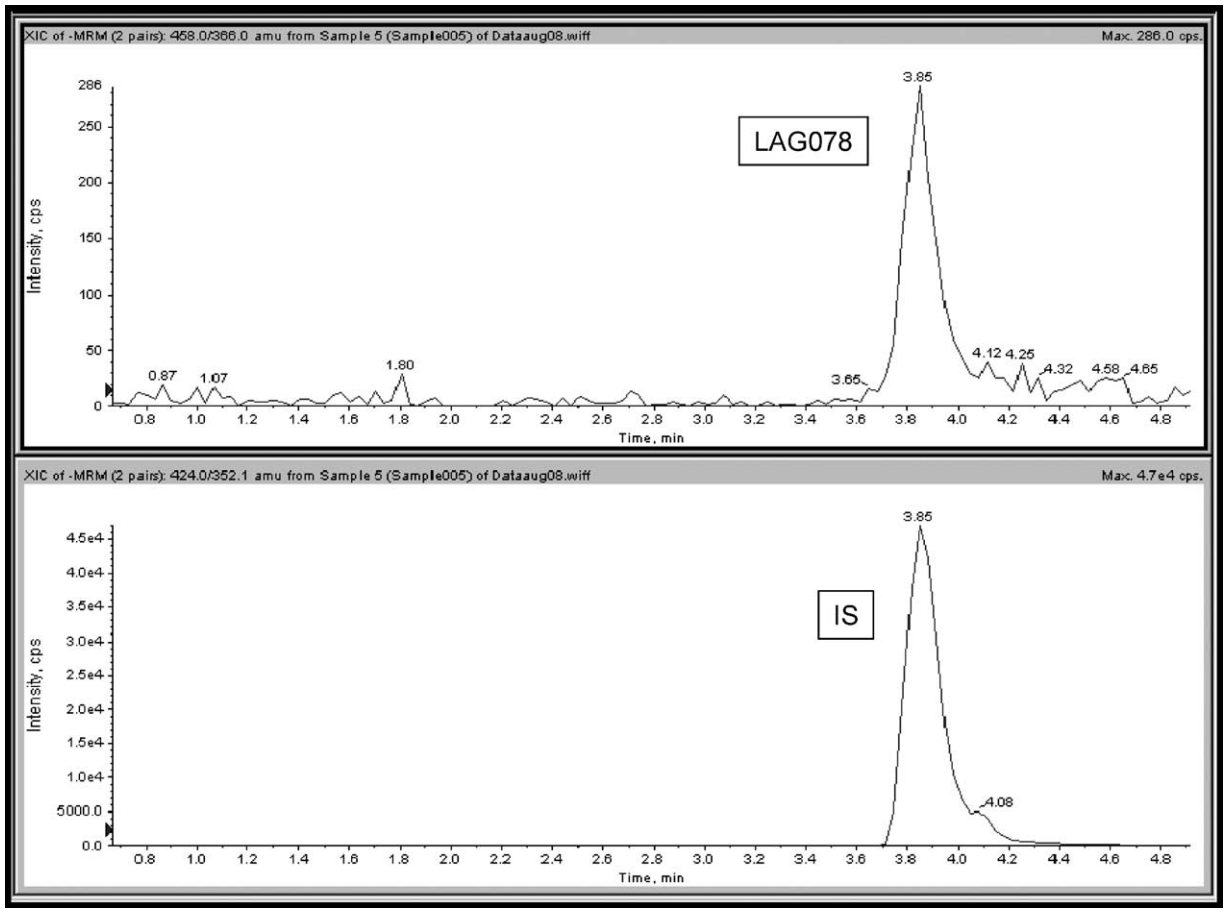

Fig. 6. LC–TIS–MS–MS ion-chromatograms in blank dog plasma spiked with LAG078 at a final concentration of 0.05 ng/ml (LLOQ) and the internal standard (7.8 ng/ml). Retention time (min) is shown on the horizontal axis and the peak intensity is shown in the vertical axis.

(7.8 ng/ml) are shown in Fig. 6. Signal to noise ratio (S/N) of the LAG078 at LLOQ was larger than 14. This value can be estimated by comparing the signal intensities at 3.85 min in Fig. 6 versus Figs. 4 and 5. Furthermore, ion chromatograms of LAG078 at the upper limit of the calibration range (50 ng/ml) and the internal standard (7.8 ng/ml) are shown in Fig. 7.

3.6. Stability

The stability of LAG078 under different conditions were studied at concentrations 0.15, 2.5, and 40 ng/ml and compared with data obtained from freshly prepared QC samples at these concentrations. Analyses were performed in triplicate and the results are displayed in Table 4. LAG078 was stable in dog plasma for at least 72 h at room temperature (25 °C). The respective mean recoveries were 83.3, 113, and 91.5% at concentrations 0.15, 2.5, and 40 ng/ml after 72 h of storage at room temperature (Table 4). The effect of freeze (−20 °C)-thaw cycles on the stability of LAG078 was studied on freshly prepared QC samples. Analogous to the room temperature experiments, no apparent loss of LAG078 was detected after three freeze-thaw cycles. The respective mean recoveries after three freeze-thaw cycles were 86.0, 102, and 93.8% of the nominal values of 0.15, 2.5, and 40 ng/ml (Table 4). No noticeable loss of LAG078

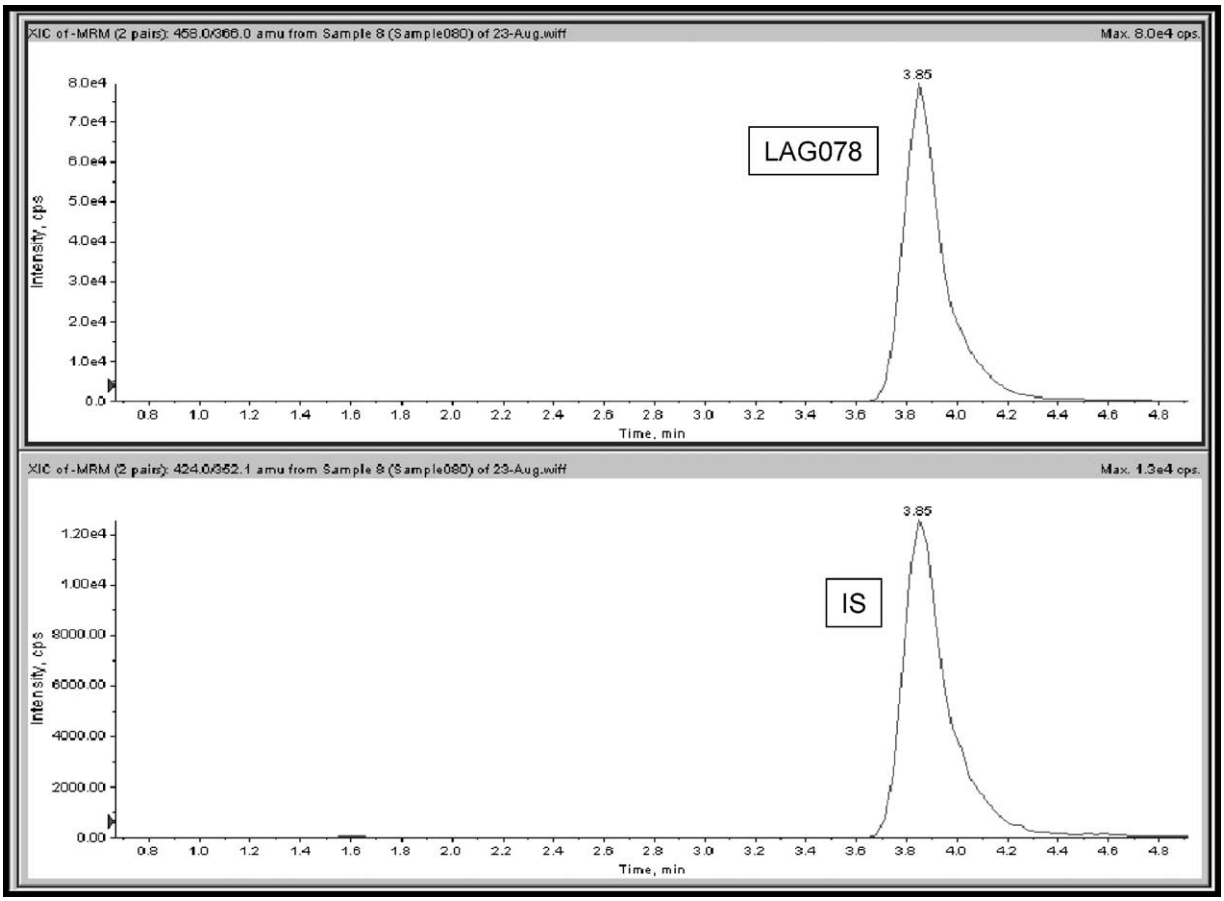

Fig. 7. LC–TIS–MS–MS ion-chromatograms in blank dog plasma spiked with LAG078 at a final concentration of 50 ng/ml (ULOQ) and the internal standard (7.8 ng/ml). Retention time (min) is shown on the horizontal axis and the peak intensity is shown in the vertical axis.

was observed after storage of freshly prepared QC samples at −20 °C for 6 weeks. The mean recoveries of the frozen samples were 92.0, 103, and 104% of the nominal values of 0.15, 2.5, and 40 ng/ml (Table 4).

3.7. Toxicokinetic application

The validated method was used to support a 13-week toxicity study in which LAG078 was orally (gavage) administered to dogs (beagle). The vehicle used during dosing was 0.5% (w/v) sodium carboxymethylcellulose, aqueous solution. Plasma samples were collected from the animals at 0.5, 1, 2, 6, and 24 h post-dose on days 1 and in week 11. Toxicokinetic parameters were calculated using the plasma concentrations obtained from 0 to 24 h samples and shown in Table 5. The area under the plasma concentration–time curves (AUC) and standard deviation of the AUC values of the analytes were calculated from 0 to 24 h post-dose by the trapezoidal rule [14]. The dose–response plots are shown in Fig. 8. The results (Fig. 8; Table 5) clearly show that LAG078 exposure increased with dose in a dose proportional manner in both male and female dogs. No consistent gender difference in exposure was observed. Repeated daily dosing for 11 weeks resulted in no compound accumulation in the animals.

Table 4
Stability data for LAG078 in dog plasma

Storage period and storage conditions	Nominal concentration (ng/ml)	Measured concentration (ng/ml)			Mean concentration (ng/ml)	Mean recovery (%)
		1st replicate	2nd replicate	3rd replicate		
0 h, 25 °C	0.15	0.131	0.148	0.142	0.140	93.3
	2.5	2.33	2.47	2.32	2.37	94.8
	40	38.0	36.4	37.2	37.2	93.0
48 h, 25 °C	0.15	0.126	0.13	0.126	0.127	84.7
	2.5	2.8	2.73	2.75	2.76	110
	40	36.5	36.3	42.8	38.5	96.3
72 h, 25 °C	0.15	0.123	0.124	0.128	0.125	83.3
	2.5	2.83	2.85	2.78	2.82	113
	40	37.2	35.9	36.7	36.6	91.5
3 freeze-thaw cycles	0.15	0.132	0.126	0.128	0.129	86.0
	2.5	2.50	2.63	2.49	2.54	102
	40	37.4	38.0	37.2	37.5	93.8
6 weeks, −20 °C	0.15	0.132	0.148	0.134	0.138	92.0
	2.5	2.70	2.63	2.42	2.58	103
	40	41.6	39.2	43.6	41.5	104

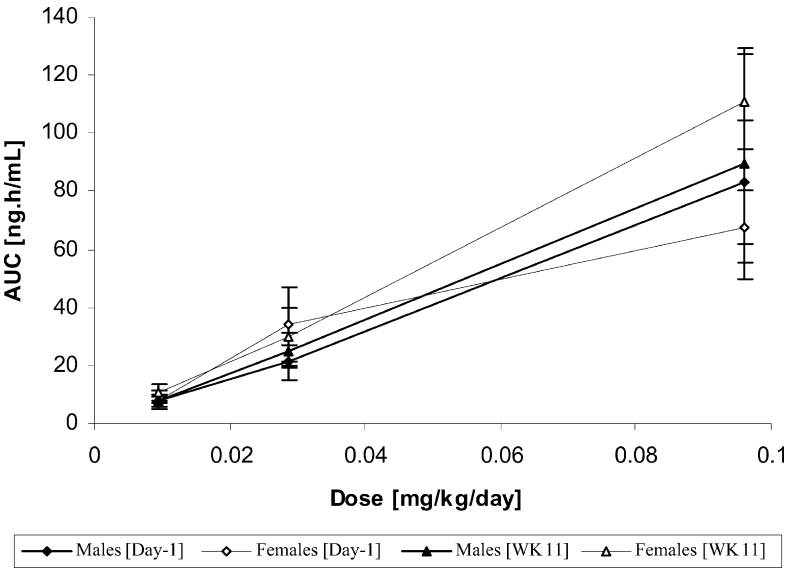

Fig. 8. Dose–response curves constructed by plotting the mean $AUC_{(0–24\,h)}$ of LAG078 vs. dose.

Table 5
Mean (±S.D., $n = 3$) toxicokinetic parameters of LAG078 in dog plasma

TK parameters	Day 1 (week 1)		Week 11	
	Male	Female	Male	Female
Group 2—(0.01 mg/kg per day)				
$AUC_{(0-24\,h)}$ (ng h/ml)	8.12 ± 0.938	7.89 ± 1.89	8.09 ± 2.95	10.4 ± 2.85
C_{max} (ng/ml)	1.37 ± 0.555	1.55 ± 0.352	1.08 ± 0.132	2.31 ± 0.617
t_{max} (h)	1 ± 0.87	1.50 ± 0.87	1.67 ± 0.58	1.17 ± 0.76
Group 3—(0.03 mg/kg per day)				
$AUC_{(0-24\,h)}$ (ng h/ml)	21.0 ± 6.16	34.1 ± 12.6	25.1 ± 6.12	29.6 ± 10.0
C_{max} (ng/ml)	6.89 ± 1.96	9.30 ± 4.59	5.72 ± 1.34	6.71 ± 2.07
t_{max} (h)	0.83 ± 0.29	1.17 ± 0.76	0.67 ± 0.29	1.17 ± 0.76
Group 4—(0.10 mg/kg per day)				
$AUC_{(0-24\,h)}$ (ng h/ml)	82.9 ± 21.3	67.6 ± 12.4	89.5 ± 39.7	111 ± 16.2
C_{max} (ng/ml)	30.4 ± 17.0	24.2 ± 15.7	19.6 ± 13.0	24.4 ± 7.07
t_{max} (h)	0.83 ± 0.29	0.67 ± 0.29	1.67 ± 0.58	1.33 ± 0.58

4. Conclusions

A LC–MS–MS method has been developed and validated for the quantification of LAG078 in dog plasma. The method exhibited ruggedness and was successfully used in a 13-week safety study. Excellent linearity was observed over the concentration range of 0.05–50 ng/ml of LAG078. Use of automated liquid–liquid extraction procedure in 96-well format for sample preparation followed by LC separation using gradient on a short column provided a high throughput method. The application of negative turbo-ionspray followed by MRM mode of detection resulted in excellent selectivity of the method.

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