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High-throughput determination of ultra-low concentrations of LAG078, a lipid modulator, in human plasma

Tapan K. Majumdar*, Ray Bakhtiar[1], Cindy Chen, Luis Ramos, Francis L.S. Tse

Department of Preclinical Safety, Novartis Institutes for Biomedical Research, Building 405, Room 228, One Health Plaza, Novartis, East Hanover, NJ 07936, USA

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Abstract

A high throughput method with ultra-low level quantification limit (10 pg/ml) was developed and validated for the quantitative determination of LAG078, a lipid modulator, in human plasma to support clinical studies employing low doses of the compound. The method consisted of reverse phase chromatographic separation of the analyte from plasma extract followed by electrospray ionization (ESI) in the negative ion mode and tandem mass spectrometry in the multiple reaction monitoring mode (MRM). 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. Optimised chromatographic separation in a short and high-resolution column (50 mm × 2.0 mm i.d., 3 μm particle size) coupled with MRM mode of detection yielded clean chromatograms with minimal signal suppression. The standard curve was linear ($r = 0.996$) within the concentration range of 0.01 (lower limit of quantification) to 50 ng/ml using 0.5 ml of human plasma. The accuracy of the method varied from 95–101% with a precision (CV) of 5.29–13.2% over the concentration range. The method was simple and rapid.

Keywords: LAG078; Lipid modulator; LDL; Turbo-ionspray; Tandem mass spectrometry; Lp(a)

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 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 of thyroid hormones (L-T3, L-T4) in lipid metabolism [2,3]. It has been observed that hypothyroidism in patients result in high plasma levels of cholesterol and low-density lipoprotein (LDL) leading to increased risk of atherosclerosis [4,5]. On the other hand, hyperthyroid patients have decreased levels of LDL.

* Corresponding author. Tel.: +1 862 778 3582; fax: +1 862 778 6076.
E-mail address: tapan.majumdar@pharma.novartis.com (T.K. Majumdar).
[1] Present address: Merck and Co., Rahway, NJ 07065, USA.

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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 effective 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 promising lipid lowering activity in the normocholesterolemic human model while demonstrating no adverse cardiac side effects in the rat cardiovascular safety model (unpublished data). This report describes a high-throughput LC/MS/MS method for the quantitative determination of LAG078 in human plasma with a LLOQ (lower limit of quantification) of 10 pg/ml. Pharmacokinetic parameters from a dose-escalation study in healthy human subjects are also discussed.

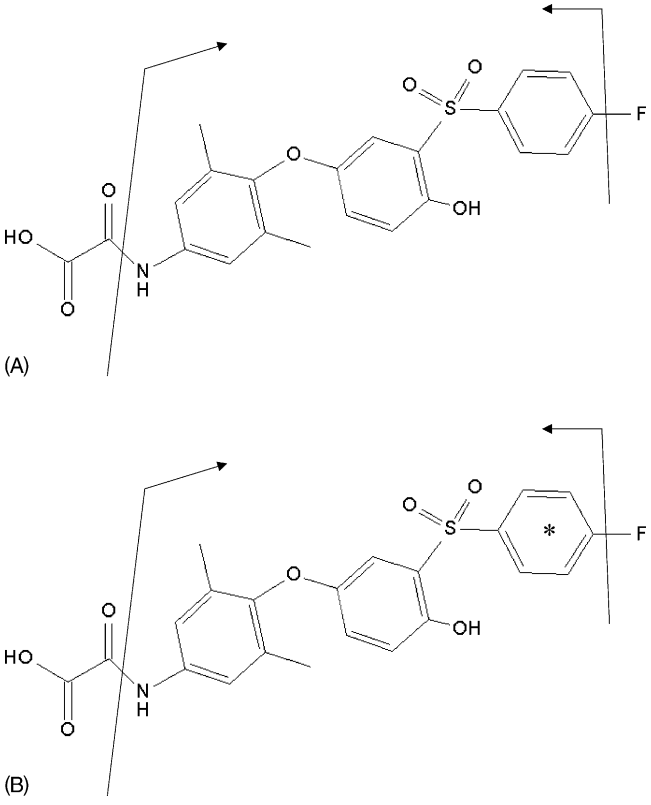

Fig. 1. Structures of (A) LAG078 and (B) the internal standard [$^{13}C_6$]-LAG078. The ^{13}C-labeled aromatic ring is shown by an asterisk in Fig. 1B. The arrow refers to the resulting bond cleavages during MS/MS to produce the most abundant product ion (*m/z* 366 for LAG078 and *m/z* 372 for the internal standard).

2. Experimental

2.1. Chemicals

All the solvents and chemicals were of HPLC or analytical grade and used without further purification. Methanol, ethanol, acetonitrile, and *tert*-butyl methyl ether (MTBE) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetic acid (glacial) was purchased from Fluka (Milwaukee, WI, USA). Water was purified using Milli-Q system from Millipore Corporation (Bedford, MA, USA). Blank human plasma (anticoagulant: disodium EDTA) was obtained from Biological Speciality Corporation (Colmar, PA, USA). The protein precipitation solvent was prepared by mixing 900 ml of acetonitrile, 100 ml of ethanol and 1.0 ml of glacial acetic acid. The reconstitution solvent was prepared by mixing 750 ml of water, 250 ml of acetonitrile, and 1.0 ml of glacial acetic acid. LAG078 ($C_{22}H_{18}FNO_7S$, average MW = 459.5 and monoisotopic mass = 459.1) reference standard and [$^{13}C_6$]-LAG078 ($^{13}C_6C_{16}H_{18}FNO_7S$, average MW = 465.4 and monoisotopic mass = 455.1) 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 (0.1 mg/ml) of LAG078 was prepared by dissolving 10.42 mg of LAG078 monohydrate (free acid/mono-hydrate ratio of 0.96) in 100 ml methanol. Working solutions of the reference standard were prepared at concentrations 0.1, 0.24, 2.0, 20, 100, 250, and 500 ng/ml by serial dilution of the stock solution in methanol. A 50 μl aliquot of each of the working solutions was spiked into 0.5 ml of blank human plasma to prepare the calibration standards at plasma concentrations of 0.01, 0.024, 0.20, 2.0, 10, 25, and 50 ng/ml, respectively. A stock solution (0.1 mg/ml) for the QC samples of LAG078 was prepared by separately weighing the solid compound and dissolving in methanol. QC

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

Time (min)	Mobile phase A (%)	Mobil phase B (%)	Flow rate (ml/min)
0.0	75	25	0.25
1.0	75	25	0.25
2.5	10	90	0.25
3.0	10	90	0.25
3.5	75	25	0.25
4.0	75	25	0.25

samples were prepared at plasma concentrations of 0.0102, 0.0246, 0.176, 8.80, 22.0, and 44.0 ng/ml by spiking working solutions of LAG078 in pooled blank human plasma and stored in small aliquots (2 ml) at −20 °C pending analysis. A stock solution of the internal standard (IS) [$^{13}C_6$]-LAG078 was prepared in methanol at 0.1 mg/ml. A working solution (20 ng/ml) of the IS was prepared by diluting the stock solution in methanol. A 50 μl aliquot of the working solution was spiked into each human plasma sample (0.5 ml) during the analysis to yield a final concentration of 2 ng/ml in human plasma.

2.3. Liquid chromatography

The chromatographic separations were performed using a Polaris C_{18}-A (50 mm × 2.0 mm i.d., 3 μm particle size) column (Metachem Technologies, Inc., Torrance, CA, USA). The column temperature was maintained at 40 °C. 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 utilised with the LC system. The autosampler was a HTS PAL (Leap Technologies, Carrboro, NC, USA). Table 1 summarises the chromatographic separation conditions performed using a gradient of solvent A (0.1% acetic acid in water) and solvent B (90% acetonitrile/10% methanol, v/v). The injection volume was 30 μl and the column pressure was approximately 1000 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 20 eV for both LAG078 and the IS. Nitrogen was used as the collision gas at setting 8. The electron multiplier voltage was maintained at 2.3 kV. The MS/MS product ion transitions of *m/z* 458 (precursor ion) → 366 (dwell time = 1.6 s) and *m/z* 464 (precursor ion) → 372 (scan time = 1.2 s) were used for LAG078 and the internal standard ([$^{13}C_6$]-LAG078), respectively. Fig. 2A and B 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 (50 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 50 μl aliquot of methanol was added to all the other wells. Blank human plasma (0.5 ml) was added to each well containing the calibration standards and blanks. The QC samples were thawed to room temperature. A 0.5 ml aliquot of each QC sample was added to specific wells in the 96-well plate. Working solution (50 μl) of the internal standard was added to each well except the wells designated for blanks where a 50 μl aliquot of methanol 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 0.5 ml aliquot of protein precipitation solvent (Section 2.1) to each well. The plate was vortexed for 5 min. Liquid–liquid extraction was performed by adding a 0.5 ml aliquot of *tert*-butyl methyl ether (MTBE) to each well. The plate was then vortexed for 1 min and then centrifuged at approximately 1000 × *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 liquid–liquid extraction was repeated one more time using 0.5 ml of MTBE to the aqueous layer in each well as described above. The organic layer from each well was transferred to the 96-well plate containing the extracts from the first extraction. The pooled organic extract in the square well block was evaporated using the flow of dry nitrogen gas at 40 °C in a 96-channel solvent evaporator (TurboVap 96 Concentration Workstation, Zymark Corporation, Hopkinton, MA, USA). The samples were reconstituted by adding a 100 μl aliquot of reconstitution solvent (Section 2.1) 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 (30 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 TurboQuan software, version 1.0 for Apple Macintosh Com-

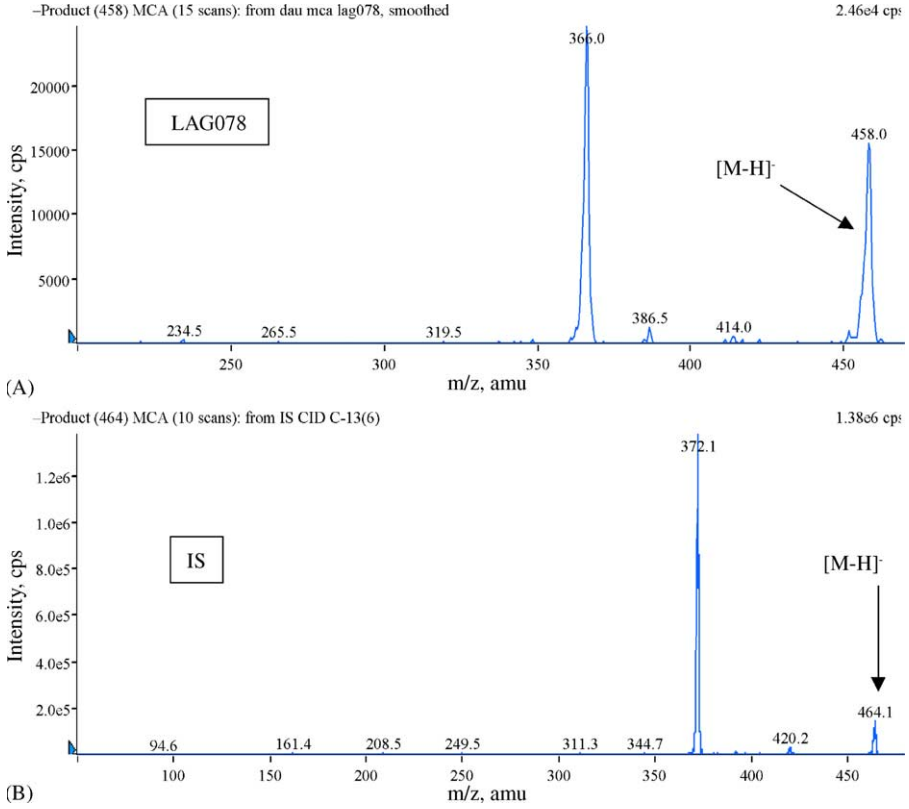

Fig. 2. The upper panel is a representative full scan TIS/MS/MS spectrum of LAG078 (collision energy −20 eV and collision gas pressure at setting 8) showing the product ion at *m*/*z* 366 and the precursor ion at *m*/*z* 458. The lower panel is a representative full scan TIS/MS/MS spectrum of the internal standard [$^{13}C_6$]-LAG078 (collision energy −20 eV and collision gas pressure at setting 8) showing the product ion at *m*/*z* 372.1 and the precursor ion at *m*/*z* 464.1. Mass to charge ratio (*m*/*z*) is shown on the horizontal axis and the ion intensity is shown in the vertical axis.

puter (Sciex Corporation, Toronto, Canada). Statistical analysis was performed using Microsoft Excel, version 6.0 (Microsoft Corporation, Redmond, WA, USA). The calibrators 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

Table 2
Summary of calibration curve obtained for the analysis of LAG078 in human plasma

Analysis day	Nominal concentration (ng/ml)							Slope (a)	y-Intercept (b)	Correlation coefficient (r)
	0.010	0.024	0.200	2.0	10.0	25.0	50.0			
	Back-calculated concentration (ng/ml)									
Day 1	0.0111	0.0207	0.238	2.11	9.13	23.2	49.5	0.529	0.0493	0.994
	0.00985	0.0212	0.222	1.98	10.7	22.7	50.5			
Day 2	0.0105	0.0240	0.225	1.87	9.47	25.8	46.1	0.561	0.0179	0.997
	0.00933	0.0241	0.227	1.93	9.84	22.6	53.1			
Day 3	0.0097	0.0224	0.227	1.89	9.08	25.3	47.2	0.378	0.0172	0.997
	0.0104	0.0245	0.204	1.97	9.54	24.9	56.8			
Mean	0.0101	0.0228	0.224	1.96	9.63	24.1	50.5	0.489	0.0281	0.996
±S.D.	0.000641	0.00165	0.0111	0.0859	0.596	1.41	3.94	0.0977	0.0183	0.00173
CV (%)	6.35	7.24	4.96	4.38	6.19	5.85	7.80	20.0	65.1	0.174
n	6	6	6	6	6	6	6	3	3	3
Accuracy (%)	101	95.0	112	98.0	96.3	96.4	101	–	–	–

Calibration parameters a and b of the calibration function $y = ax + b$ and correlation coefficient r on each day of the validation.

area ratios and the regression equation of the calibration curve.

3. Results and discussion

The analytical approach used in this method satisfied the need for a high throughput assay for LAG078 to support clinical studies. The LC/MS/MS of LAG078 exhibited a good sensitivity with linear response within the concentration range of 0.01–50 ng/ml in the negative ion mode using a turboionspray (TIS) interphase. The extraction procedure was optimized to minimize the matrix interference. The chromatographic separation was optimized on a minibore (2.0 mm i.d.) short (5 cm long) and high resolution (3 μm particle size) column for the early elution of polar matrix interferences rendering minimal ion suppression typically observed during electrospray ionization [9–14]. 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. A representative MS/MS product spectrum of ion *m/z* 458 $[M-H]^-$ from LAG078 is shown in Fig. 2A. 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* 372 was formed from the precursor ion $[M-H]^-$ by the same fragmentation process as that of LAG078 and is shown in Fig. 2B.

3.1. Calibration curves

The calibration curve parameters from 3 days of validation are summarised in Table 2. The mean correlation coefficient was 0.996. 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 95.0–112% from theoretical value and indicated a good fit of the regression model over the concentration range 0.01–50 ng/ml of the calibration curve. The coefficient of variation (CV) did not exceed 7.8%.

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

Both intra-day and inter-day accuracy and precision of the method were evaluated from six replicates of QC samples of known concentrations. These QC samples were prepared at six different concentrations spread along the calibration curve range. The lowest QC sample of 0.0102 ng/ml (Table 3) was also 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 87.5 to 112% over the 0.01–44 ng/ml concentration range of LAG078; the corresponding precision ($n = 6$) varied from 0.79 to 12.1%. The inter-day mean recoveries varied from 95.0 to 101% and the corresponding precision ($n = 18$) varied from 5.29 to 13.2%.

3.3. Lower limit of quantitation

The lower limit of quantitation (LLOQ), defined as the lowest concentration on the standard curve that could be mea-

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

Analysis day	Nominal concentration (ng/ml)					
	0.0102	0.0246	0.176	8.80	22.0	44.0
Day 1 ($n = 6$)						
Intra-day mean accuracy	107	92.0	93.5	100	94.1	98.2
±S.D.	12.2	10.1	5.26	4.64	0.745	2.72
CV (%)	11.4	11.0	5.63	4.64	0.790	2.77
Day 2 ($n = 6$)						
Intra-day mean accuracy	102	110	87.5	94.1	95.8	92.9
±S.D.	12.3	5.94	4.50	4.54	5.70	3.93
CV (%)	12.1	5.40	5.14	4.82	5.95	4.23
Day 3 ($n = 6$)						
Intra-day mean accuracy	93.7	100	104	98.8	112	110
±S.D.	10.4	6.75	8.93	3.50	3.24	4.14
CV (%)	11.1	6.75	8.59	3.54	2.89	3.76
Inter-day mean ($n = 18$)	101	101	95.0	97.8	101	100
±S.D.	13.3	10.9	9.72	5.17	9.11	8.18
CV (%)	13.2	10.8	10.2	5.29	9.02	8.18

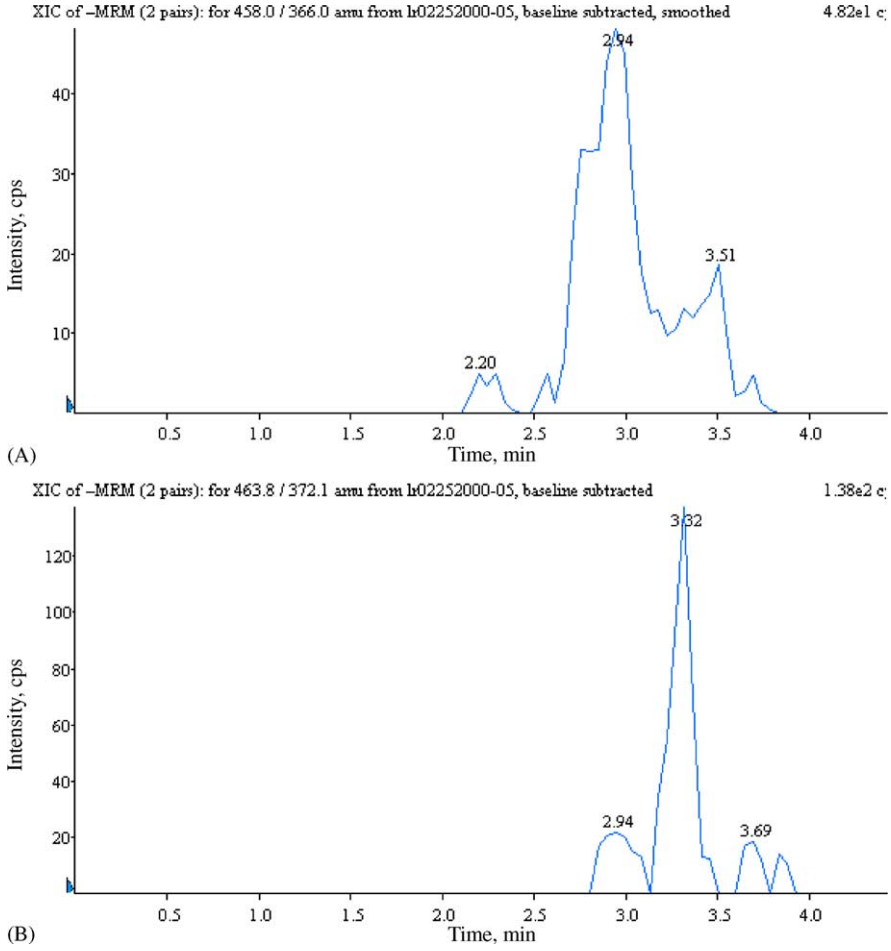

Fig. 3. HPLC/TIS/MS/MS ion-chromatograms in blank human plasma. Panel (A) is the ion chromatogram for LAG078. Panel (B) is the ion chromatogram for the internal standard. Retention time (min) is shown on the horizontal axis and the peak intensity is shown in the vertical axis.

sured with acceptable accuracy and precision, was 0.01 ng/ml using 0.5 ml of human plasma. The accuracy ($n = 18$) of determination at LLOQ was 101% with a precision of 13.2% (Table 3).

3.4. Specificity

The instrument was operated in the MRM mode to maximise the specificity and sensitivity. The fragmentation reactions of m/z 458 → 366 and m/z 464 → 372 were monitored for LAG078 and the internal standard, respectively. Representative chromatograms of extracts of blank (free of LAG078) human plasma and of the same plasma spiked with the internal standard are shown in Figs. 3 and 4, respectively. 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 approximately 2.85 and 2.90 min, respectively. It was expected that the stable isotope labeled internal standard would contribute to the MS/MS ion chromatogram of the parent compound LAG078. In this method the internal standard did contribute to the MRM channel of LAG078 at the same retention time as LAG078 as shown in Fig. 4A. This contribution was due to a small amount (0.9%) of unlabeled LAG078 present in the internal standard as isotopic impurity. It is important to keep this contribution to a minimum so that the LLOQ (lower limit of quantification) of the method was not affected significantly. In this method the plasma concentration of the internal standard was 2 ng/ml. Ion chromatograms of LAG078 at the LLOQ (0.01 ng/ml) and the internal standard (2 ng/ml) are shown in Fig. 5. From Figs. 4 and 5 it is clear that the contribution of the internal standard was very low compared to the signal intensity of LAG078 at the LLOQ level. Furthermore, ion chromatograms of LAG078 at 25 ng/ml and the internal standard (2 ng/ml) are shown in Fig. 6. Specificity of the method was investigated further by analyzing six different lots of blank human plasma samples in duplicates on each day of validation.

3.5. Stability

The stability of LAG078 under different conditions was studied at concentrations 0.0246, 22, and 44 ng/ml. Analy-

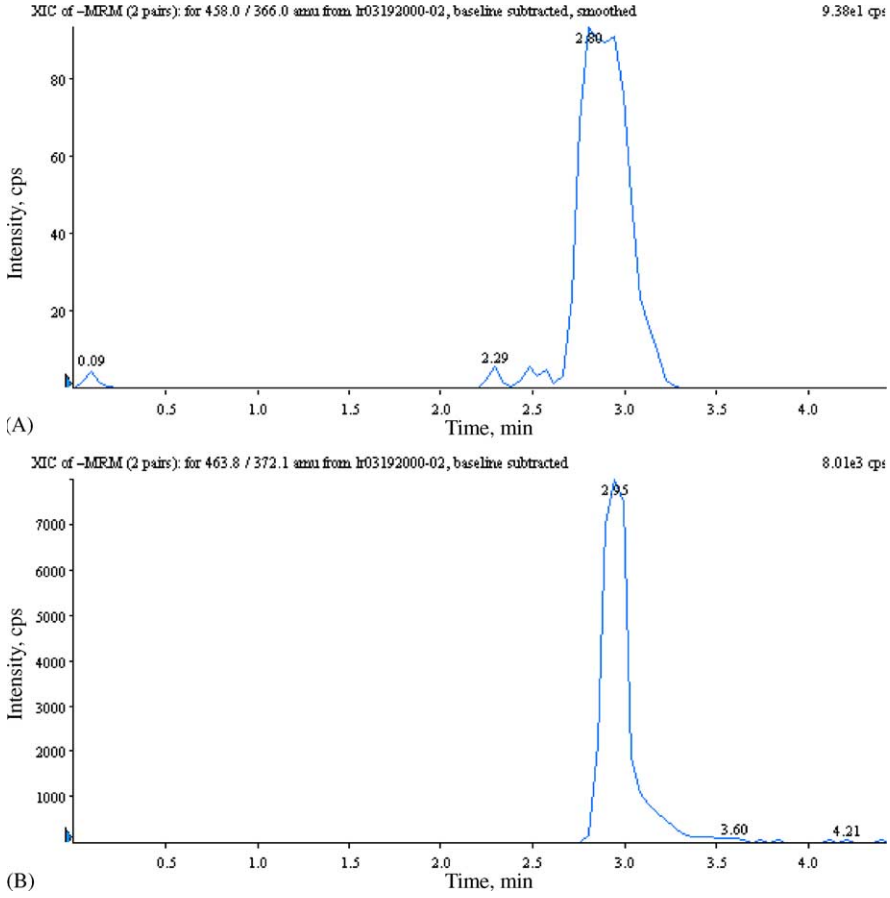

Fig. 4. HPLC/TIS/MS/MS ion-chromatograms in blank human plasma spiked with the internal standard (2 ng/ml): Panel (A) is the ion chromatogram for LAG078. Panel (B) is the ion chromatogram for the internal standard. Retention time (min) is shown on the horizontal axis and the peak intensity is shown in the vertical axis.

ses were performed in triplicate and the results are displayed in Table 4. LAG078 was stable in human plasma for at least 24 h at room temperature (25 °C). The respective mean recoveries were 91.5, 93.6, and 90.2% at concentrations 0.0246, 22.0, and 44.0 ng/ml after 24 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

Table 4
Stability data for LAG078 in human plasma

Storage period and storage conditions	Nominal concentration (ng/ml)	Recovery (%)			Mean recovery (%)
		1st replicate	2nd replicate	3rd replicate	
4 h, 25 °C	0.0246	111	114	94.7	107
	22.0	102	96.4	97.3	98.6
	44.0	84.3	91.4	103	92.9
24 h, 25 °C	0.0246	88.2	87.0	99.2	91.5
	22.0	89.5	94.5	96.8	93.6
	44.0	95.5	90.9	84.1	90.2
3 Freeze-thaw cycles	0.0246	79.3	108	97.2	94.8
	22.0	104	91.8	87.3	94.4
	44.0	94.8	93.6	92.7	93.7
3 Weeks, −20 °C	0.0246	86.5	91.3	111	96.3
	22.0	104	105	97.9	102
	44.0	89.4	93.7	96.2	93.1

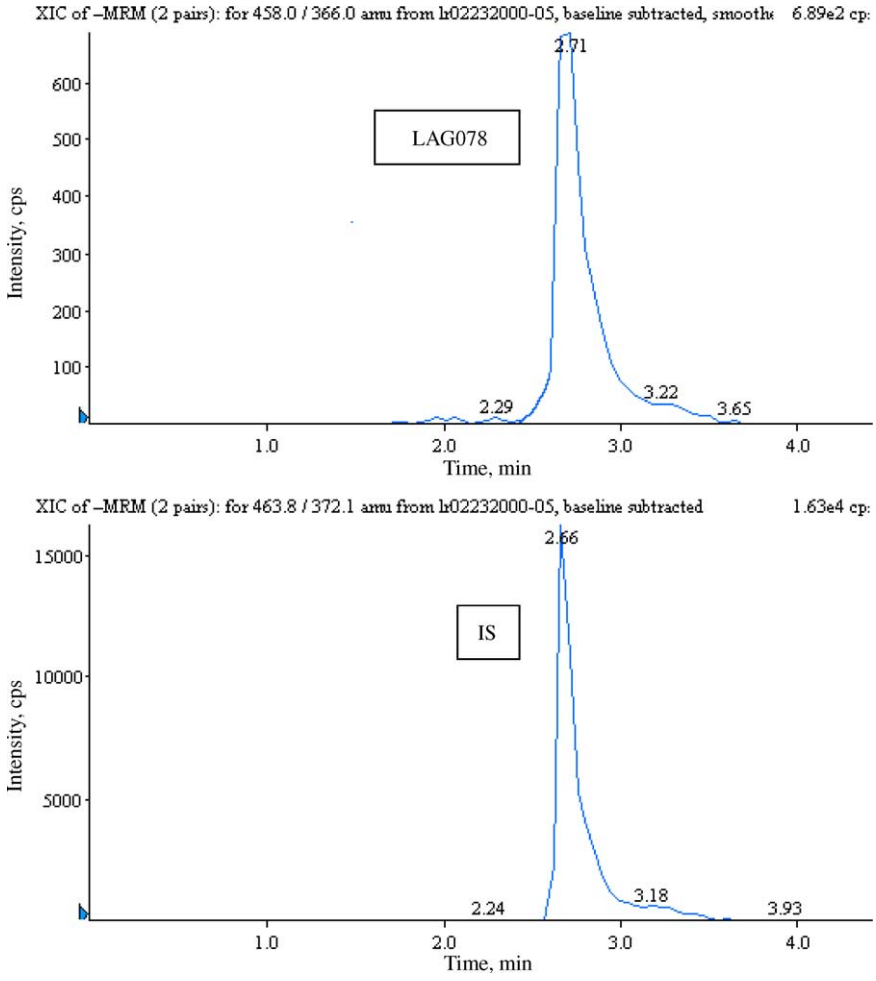

Fig. 5. HPLC/TIS/MS/MS ion-chromatograms in blank human plasma spiked with LAG078 at a final concentration of 0.01 ng/ml (LLOQ) and the internal standard (2 ng/ml). Retention time (min) is shown on the horizontal axis and the peak intensity is shown in the vertical axis.

apparent loss of LAG078 was detected after three freeze-thaw cycles. The respective mean recoveries after three freeze-thaw cycles were 94.8, 94.4, and 93.7% of the nominal values of 0.0246, 22.0, and 44.0 ng/ml (Table 4). No noticeable loss of LAG078 was observed after storage of freshly prepared QC samples at −20 °C for 3 weeks. The mean recoveries of the frozen samples were 96.3%, 102%, and 93.1% of the nominal values of 0.0246, 22.0, and 44.0 ng/ml (Table 4).

3.6. Pharmacokinetic application

The validated method was used to support a dose-escalation study in which healthy subjects ware administered orally (solution) with single doses of LAG078 at 30, 100, 200, 450, 900, and 1800 μg. Pharmacokinetic parameters were calculated using the plasma concentrations obtained from 0–24 h samples and the mean ($n = 4$) values are shown in Table 5. The mean plasma concentration-time profiles of different dose groups are shown in Fig. 7. The area under the plasma concentration–time curve (AUC) values of the analyte were calculated from 0 to 24 h postdose samples by the trapezoidal rule [15]. LAG078 was rapidly absorbed as shown by T_{max} values of 1 h or less (Table 5). The results (Table 5) showed that LAG078 exposure (in terms of both AUC and C_{max}) increased with dose in a dose proportional manner.

Table 5
Mean (±S.D., $n = 4$) pharmacokinetic parameters of LAG078 after oral administration

Dose (μg)	$AUC_{(0-24\,h)}$ (ng h/ml)	C_{max} (ng/ml)	T_{max} (h)
30	1.23 ± 0.391	0.318 ± 0.133	0.438 ± 0.125
100	5.16 ± 4.11	1.28 ± 0.976	1.38 ± 1.18
200	6.97 ± 2.46	2.47 ± 0.497	0.375 ± 0.144
450	14.6 ± 0.818	3.61 ± 1.00	0.750 ± 0.500
900	33.6 ± 7.33	9.16 ± 0.661	0.438 ± 0.125
1800	64.0 ± 14.3	18.5 ± 6.82	1.00 ± 0.00

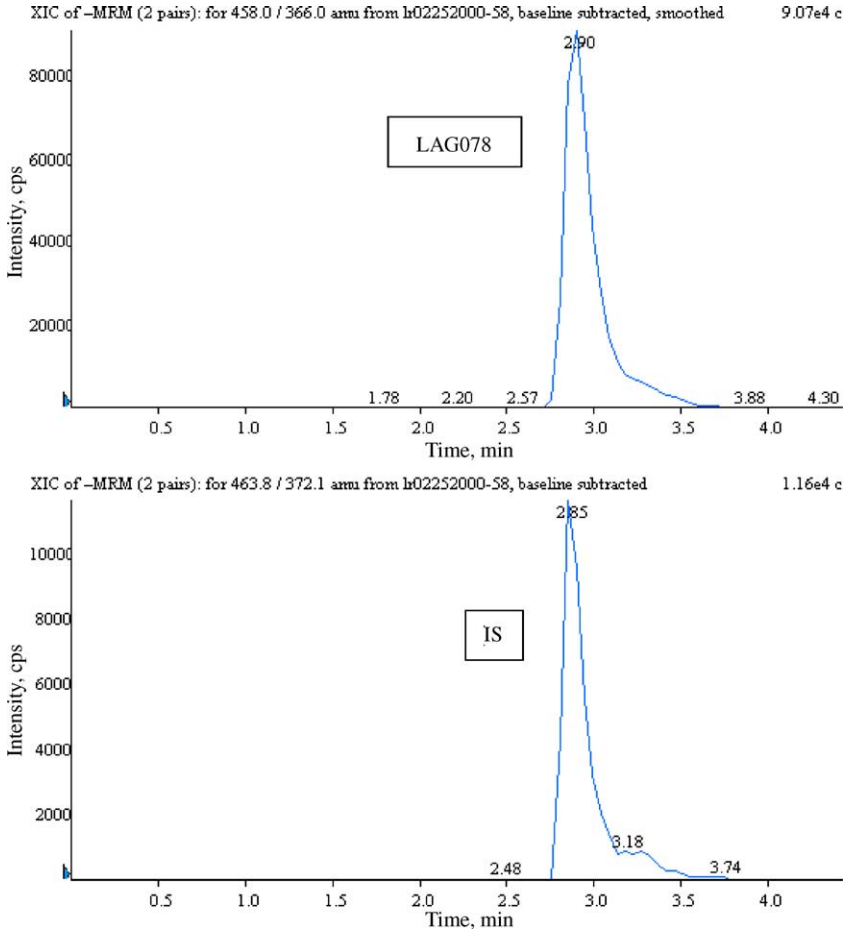

Fig. 6. HPLC/TIS/MS/MS ion-chromatograms in blank human plasma spiked with LAG078 at a final concentration of 25 ng/ml and the internal standard (2 ng/ml). Retention time (min) is shown on the horizontal axis and the peak intensity is shown in the vertical axis.

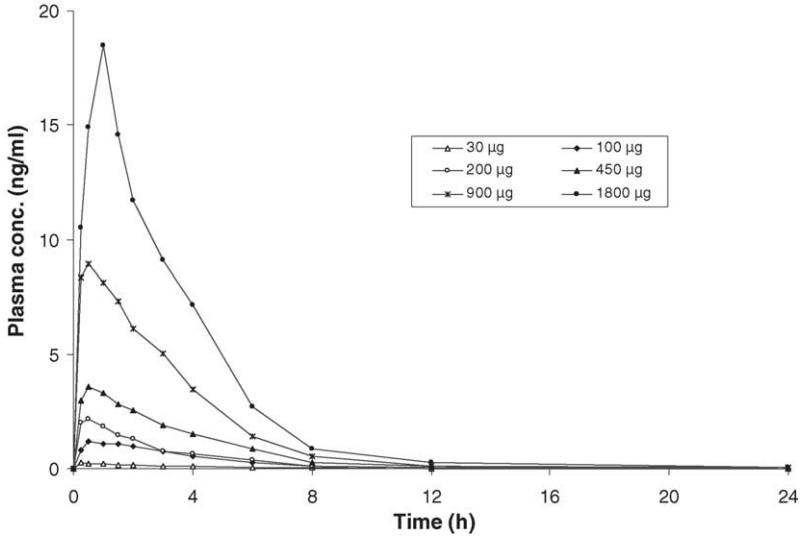

Fig. 7. Mean ($n = 4$) plasma concentration vs. time profile of LAG078 after oral administration in different dose groups.

4. Conclusions

A LC/MS/MS method has been developed and validated for the quantification of LAG078 in human plasma. The method exhibited ruggedness and was successfully used in a dose escalation study in healthy human subjects. Excellent linearity was observed over the concentration range of 0.01–50 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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