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Quantitative determination of oxytocin receptor antagonist atosiban in rat plasma by liquid chromatography–tandem mass spectrometry

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ABSTRACT

A kinetic study of atosiban was conducted following repeated intravenous administration in Wistar rats. Sample analysis was performed using liquid chromatography–tandem mass spectrometry (LC–MS/MS) following full validation of an in-house method. Eptifibatide, a cyclic peptide, was used as an internal standard (IS). The analyte and internal standard were extracted using solid phase extraction (SPE) method. Chromatographic separation was carried out using an ACE C18 5 μm 50 mm × 4.6 mm column with gradient elution. Mass spectrometric detection was performed using TSQ Quantum ultra AM. The lower limit of quantification was 0.01 μg/ml when 100 μl rat plasma was used. Plasma concentrations of atosiban were measured at 0 (pre-dose), 2, 15, 30, 45, 60, 120 min at the dosage levels of 0.125 mg/kg (low dose), 0.250 mg/kg (mid dose), and 0.500 mg/kg (high dose), respectively. Atosiban plasma concentration measured at Day 1 showed mean peak atosiban concentration (C_{max}) 0.40, 0.57, 1.95 μg/ml for low, mid and high dose treated animals and mean peak concentration on Day 28 was 0.41, 0.88, 1.31 μg/ml on Day 28 for low, mid and high dose treated animals.

1. Introduction

Pre-term labour affects 25% of human pregnancy and leads to pre-term delivery [1–4]. It is a cause of concern as premature delivery of infants (before 37 weeks of gestation) leads to complications such as neurosensory deficits, respiratory distress syndrome, low body weight, sub normal height and possible neonatal death [5]. Prevention of pre-term labour requires pharmacological inhibition of uterine contraction (tocolysis). Drugs used to suppress contractions such as magnesium sulfate, ritodrine, terbutaline, salbutamol (β agonists), nifedipine (calcium channel blocker) suffer from adverse side effects [6–10]. A recent investigation on oxytocin receptor antagonists, atosiban and barusiban indicated their high specificity for the uterus with limited or no systemic effects [11–14]. Atosiban compete with oxytocin receptors in myometrium and thus preventing the action of oxytocin in the target cells [15]. In clinical trials atosiban reduced the number of premature deliveries over seven days compared with placebo and no fetal adverse effects were seen and it is used to delay pre-term birth between 24 and 33 weeks of gestational age for 48 h.

Atosiban is a chemically synthesized nonapeptide of molecular formula $C_{43}H_{67}N_{11}O_{12}S_2$ and molecular weight 994.4 Da (Fig. 1). Pharmacokinetic studies in pregnant women showed a half-life of 18 min, clearance 42 l/h and a distribution volume of 18 litres [16]. Earlier methods used to estimate atosiban in plasma were by radioimmunoassay [17] and the reported sensitivity was 1.0 ng/l. Although immunoassay methods have superior sensitivity and throughput, they suffer from cross-reactivity due to endogenous proteins. With growing number of peptide and protein drugs getting approved for clinical trials, it is becoming important to have assay methods with improved sensitivity and selectivity. For quantification of small peptides in biological matrices tandem mass spectrometry is becoming a preferred technique [18,19]. There are no mass spectrometry based assays published so far for atosiban. Reported mass spectrometry (MS) analysis on atosiban was confined to impurity identification by fast atom bombardment tandem mass spectrometry [20] and separation of impurities by hydrophilic interaction chromatography coupled with electrospray ionisation (ESI) MS [21]. In this present study, a sensitive liquid chromatography mass spectrometry method was developed for determining plasma concentration of atosiban in a kinetic study. A simple gradient method was employed using 100% acetonitrile and 1% acetic acid with 0.05% trifluoroacetic acid in water as mobile phases. Atosiban was extracted from rat plasma using SPE technique. Positive ESI source was used for ionising atosiban and IS. Multiple reaction monitoring (MRM) method was used

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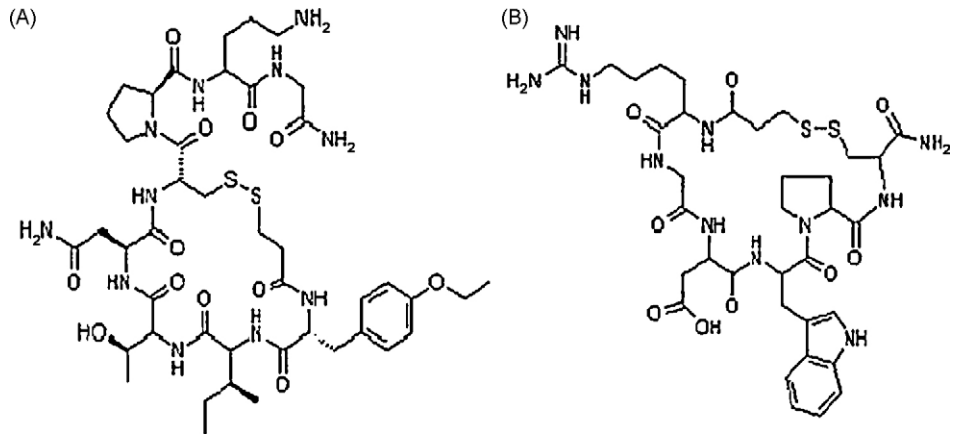

Fig. 1. Chemical structure of atosiban (A) and eptifibatide (B).

for quantification of atosiban in rat plasma for improved selectivity and sensitivity. The lower limit of quantification (LLOQ) of atosiban in rat plasma was 0.01 μg/ml. Standard calibration curves were generated at atosiban concentrations 0.01, 0.05, 0.1, 0.5, 1.0, 5.0, 10.0, 20.0 and 50.0 μg/ml. The method was validated for specificity, selectivity, recovery, accuracy, precision and stability.

2. Experimental

2.1. Materials

In-house purified atosiban (assay purity >99%) was used as standard and in-house eptifibatide (assay purity >99%) was used as IS. Stock solutions were made in water/acetonitrile (80/20, v/v) with 50 mM acetic acid. Stock solutions were stored in −20 °C for 1 week. Working standards were made from 1 mg/ml stock solution. Working standards at a concentration of 0.1, 0.5, 1.0, 5.0, 10.0, 50.0, 100.0, 200.0 and 500.0 μg/ml were prepared by using water/acetonitrile (80/20, v/v) with 50 mM acetic acid. Working standards were stored at 4 °C.

2.2. Animals and treatment

Female Wistar rats were chosen for kinetic assessments, since atosiban is indicated for delaying imminent pre-term birth in pregnant women. Twenty-seven animals were used for kinetic measurements. The animals had free access to water (deep borewell water passed through activated charcoal filter and exposed to UV rays) and standard diet (Ssniff rats pellet food manufactured by Ssniff Spezialdiäten GmbH., Ferdinand-Gabriel-Weg 16, D-59494 SÖest, Germany) and were kept under standard conditions (12-h day/night cycles, temperature 20–23 °C, humidity ~65%). The female rats (9 animals per group) were administered intravenously with 0.125 (low), 0.250 (mid) and 0.500 (high) mg/kg/day of atosiban formulation at an equivolume of 5 ml/kg/day. The treatment was continued for 28 consecutive days approximately at the same time each day. The doses were selected based on a pilot study. A vehicle treated control group was also included. On Day 1 and Day 28 of treatment, blood samples were collected at 0 (pre-dose), 2, 15, 30, 45, 60 and 120 min post-dose from three rats at a time in a staggered manner, and plasma was immediately separated by centrifugation. Heparinised plasma samples were stored at −85 °C until they were analysed.

2.3. Methods

HPLC method described here for analysis of atosiban is based on previous experience of the group analyzing insulin analogs (manuscript under review). LC–MS/MS analyses were performed on a TSQ Quantum Ultra AM mass spectrometer (Thermo, USA) coupled with a high performance liquid chromatography (Prominence, Shimadzu, Japan) equipped with LC-20AD binary pump, DGU-20A3 degasser, CTO 20AC column oven and SIL-20AC autosampler. ACE C18, 5 μm 50 mm × 4.6 mm, column (Advanced chromatography technologies, Aberdeen, Scotland) with pore size 300 Å was used for the study. A gradient method was used with 1% acetic acid and 0.05% trifluoroacetic acid in water (A) and 100% acetonitrile (B). Atosiban was eluted by performing gradient elution from 20%B to 90%B over 5 min. The column was kept at 90% B until 6.0 min and reverted to original conditions by 6.01 min. The column was re-equilibrated under isocratic conditions until 9.0 min. The injection volume was 5 μl and the injection port was washed with 1 ml of mixture of water/acetonitrile (80/20, v/v) to prevent carryover. The flow rate was 500 μl/min and the column temperature was maintained at 40 °C. Divert valve in mass spectrometer was used to divert the column effluent to waste after 6.0 min to prevent matrix components contaminating the ion source.

MRM method was chosen for quantifying atosiban in rat plasma using transitions 994.300 → 212.070; 994.300 → 286.060. Eptifibatide was used as an IS and the transition used was 832.420 → 242.100. The capillary voltage was kept at 3200 V. Capillary and vapouriser temperatures were 275 and 250 °C, respectively. Sheath gas and auxiliary gas were kept at 50 (arbitrary units) for desolvation. Atosiban precursor ion 994.3 was fragmented at collision energy 39 V and at a collision pressure 1.5 mTorr. Tube lens was kept at 150 V. The scan width and scan time were 0.020 and 0.2 s, respectively. The mass spectrometric data were processed using LCQUAN software (version 2.5).

2.4. Method of extraction

Extraction method for atosiban and eptifibatide from plasma was based on the method developed for insulin analogs (manuscript under review). Waters extraction manifold and Oasis HLB cartridges of 1 cc (30 mg) were used for extraction of atosiban and IS in heparinised rat plasma. The cartridges were conditioned with 1 ml methanol followed by 1 ml water. The cartridges were loaded with 100 μl rat plasma spiked with atosiban and IS. Spiked plasma sample was allowed to pass through the extraction cartridge under vacuum. The cartridge was then washed with 1 ml water. Atosiban and IS were eluted using 300 μl of a mixture of

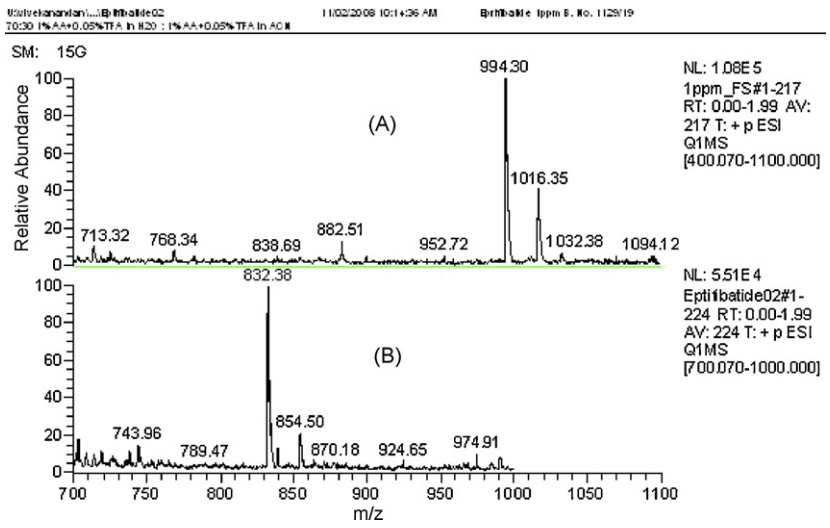

Fig. 2. Full scan electrospray ionisation mass spectra of atosiban (A) and eptifibatide (B) standards at 1 μg/ml concentration.

water/acetonitrile (70/30, v/v) with 1% formic acid. Extracted samples were transferred into a HPLC vial for LC–MS/MS analysis.

2.5. Kinetic data analysis

Kinetic parameters such as T_{max}; time at which maximum plasma concentration was observed, AUC; area under the curve, AUC_{last}; area under the curve from time 0–time t were calculated using non-compartmental analysis program of WinNonlin® software (Version 5.2.1). Since blood samples were collected from 3 of 9 rats in a group, at a time in a staggered way, descriptive statistics were performed to calculate the mean concentration ($N = 3$) at each time point prior to the non-compartmental analysis. WinNonlin model no. 201 (Plasma data, Bolus IV administration) was used for the analysis. Conditional substitution (before T_{max}: 0; after T_{max}: missing; first of consecutive after T_{max}: LLOQ/2; after first of consecutive after T_{max}: missing) for non-numerical values was adopted during non-compartmental analysis of the mean data. Linear/Log trapezoidal method was used for AUC_{last} calculations and the time for the peak plasma concentration (T_{max}) was directly taken from the observed values.

3. Results and discussion

Full scan electrospray ionisation mass spectra of atosiban and IS given in Fig. 2 show peaks corresponding to $[M+H]^+$ and corresponding sodiated adducts. Product spectrum of atosiban was obtained by optimising the collision energy (0–70 V) and collision pressure (0–2 mTorr). Fragmentation of atosiban at collision pressure 1.5 mTorr and collision energy 39 V gave consistent fragment peaks at 212 and 286 Da (Fig. 3). MRM method was chosen for quantifying atosiban for improved selectivity. Eptifibatide, a cyclic peptide of molecular weight 832 Da was chosen as an IS for quantification experiments. Atosiban and IS were extracted from rat plasma using Oasis HLB cartridges. For small animal non-clinical study 100 μl rat plasma was used.

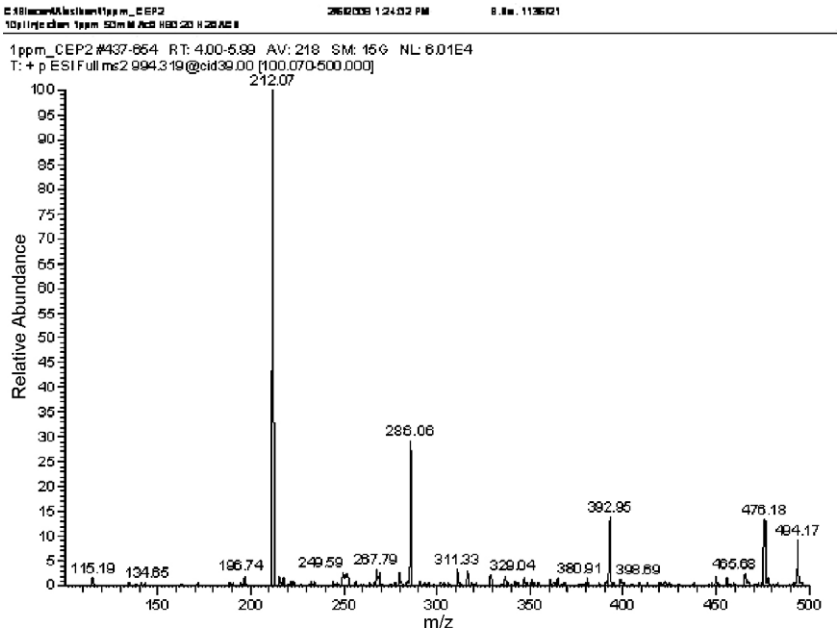

Fig. 3. Product ion mass spectrum of in-house atosiban standard recorded at 39 V collision energy.

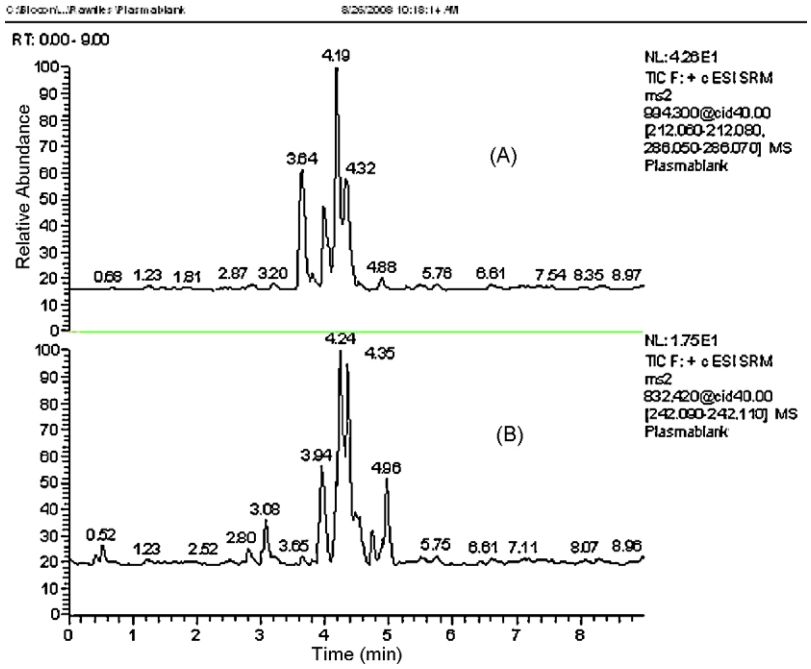

Fig. 4. Multiple reaction monitoring ion chromatograms of blank rat plasma (drug and internal standard free) for atosiban (A) and eptifibatide (B).

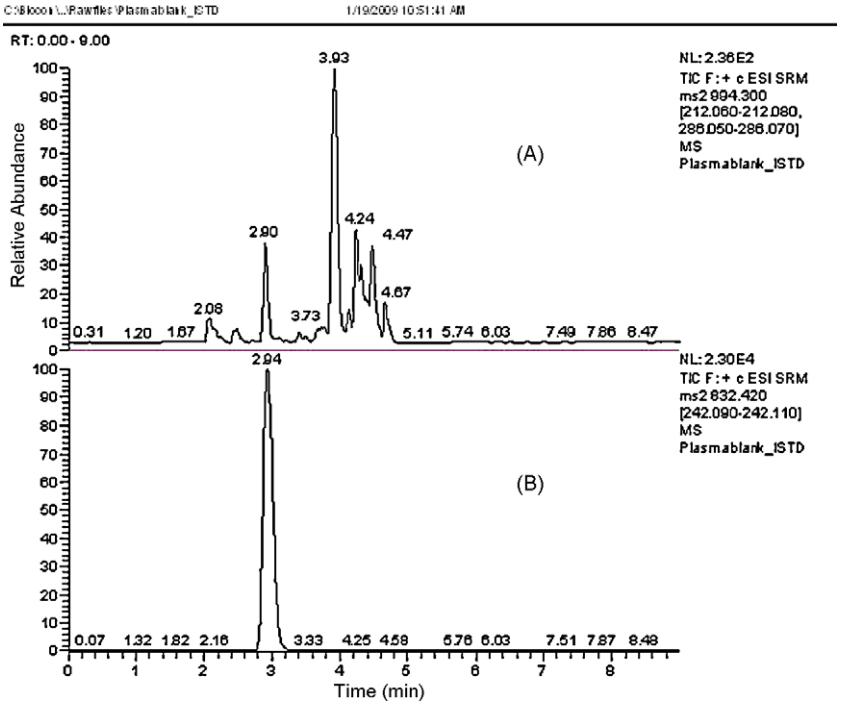

Fig. 5. Multiple reaction monitoring ion chromatograms indicating low level of interfering signals for atosiban (A) when rat plasma is spiked with 5 μg/ml of eptifibatide (B).

Table 1
Precision and accuracy of atosiban in rat plasma.

Conc. (μg/ml)	*n*	Within-batch			Between-batch		
		Mean ± SD	Precision (%)	Accuracy (%)	Mean ± SD	Precision (%)	Accuracy (%)
0.01	5	0.01 ± 0.001	8.94	100.00	0.001 ± 0.000	8.17	100.83
0.1	6	0.114 ± 0.001	0.87	114.00	0.098 ± 0.006	6.12	98.00
0.5	6	0.521 ± 0.017	3.19	104.27	0.503 ± 0.014	2.71	100.67
10.0	6	10.773 ± 0.365	3.39	107.73	10.094 ± 0.558	5.53	100.94
50.0	6	54.353 ± 0.734	1.35	108.71	53.076 ± 0.374	0.70	106.15

n = number of plasma replicates.

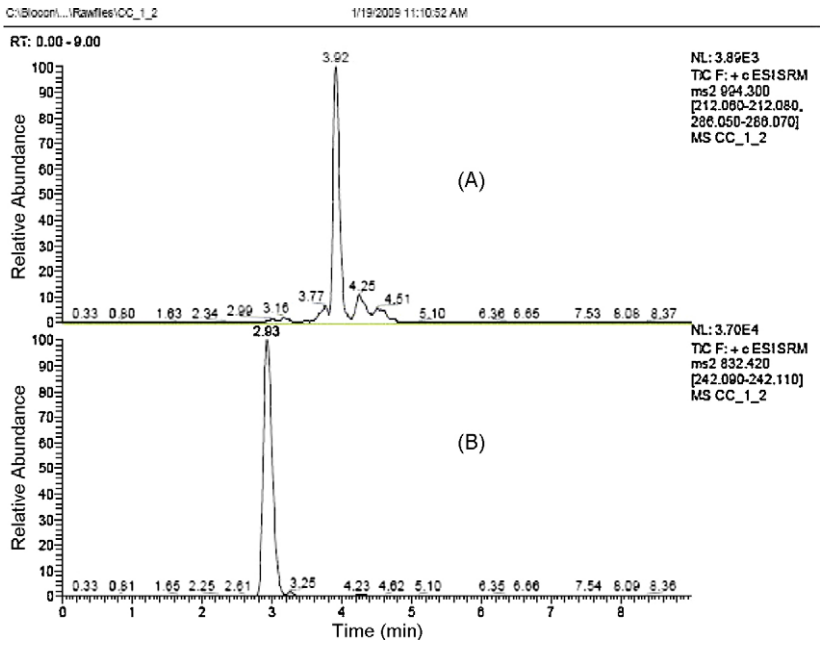

Fig. 6. Multiple reaction monitoring ion chromatogram of 0.01 μg/ml atosiban (A) and 5 μg/ml of eptifibatide (B) spiked and extracted from rat plasma.

3.1. Bioanalytical method validation

3.1.1. Calibration curves

Standard calibration curve was constructed using a double blank (extracted plasma samples without IS), blank (extracted plasma sample with IS) and nine standards along with 5 μg/ml of IS spiked and extracted from rat plasma at 0.01, 0.05, 0.1, 0.5, 1.0, 5.0, 10, 20.0 and 50.0 μg/ml concentrations. To obtain good reproducibility for MRM transition area ratio was obtained from two prominent product ions from atosiban [212.070] + [286.060] and only [242.10] ion from IS. Calculations were done using linear regression analysis with $1/x^2$ weighting using LCQUAN for atosiban. The acceptance criteria for calculated calibration standard concentrations for atosiban in rat plasma were 15% from the nominal value. The mean linear regression equation of the calibration curve for the analyte was $y = 0.00268315 + 7.66597x$, where y was the peak area ratio of the analyte (994.300 → 212.070 + 994.300 → 286.060) to IS (832.420 → 242.100) and x was the concentration of the analyte. The correlation coefficient was 0.9966. The results show linear fit from 0.01 to 50 μg/ml and found to be suitable for generating acceptable data for atosiban in rat plasma.

3.1.2. Extraction recovery

The purpose of the recovery study was to evaluate the efficiency of the SPE process. The recovery was determined spiking rat plasma samples at two different standard concentration (0.1 and 50.0 μg/ml, $n = 10$) and at one concentration (5 μg/ml, $n = 5$) for IS. Recovery of atosiban and IS were determined from the ratio of the normalised mean peak area of extracted samples to the mean peak area of the reference samples. The extraction recovery of atosiban was 71.0% (RSD: 5.67%) on an average and for IS was 99.5% (RSD: 7.43%).

3.1.3. Specificity and selectivity

Specificity and selectivity of the method were evaluated by extracting and analysing six individual lots of blank rat plasma with and without atosiban. Pooled rat plasma was used as the blank matrix throughout the validation. No significant interference was observed at the retention time of the peak for atosiban at 3.92 min (Fig. 4A) and for IS at 2.93 min (Fig. 4B) when extracted rat plasma samples were analysed. MRM chromatograms of plasma blank spiked with IS (Fig. 5B) indicate no significant interference to atosiban at 2.93 min (Fig. 5A). Although no separate experiments were carried out for limit of detection, based on signal to noise ratio

Table 2
Stability data of atosiban performed with five replicates.

Sample concentration (μg/ml)	Concentration found (μg/ml) ± SD	Precision (%)	Accuracy (%)
Short term stability (4 h)			
0.5	0.498 ± 0.011	2.27	99.68
50.0	52.447 ± 2.40	4.57	104.89
Freeze–thaw stability (3 cycles)			
0.5	0.448 ± 0.013	2.99	89.70
50.0	45.694 ± 3.23	7.06	91.30
Autosampler stability (72 h)			
0.5	0.536 ± 0.054	10.23	107.20
50.0	54.033 ± 3.16	5.85	108.06
Long-term stability at −85 °C (30 days)			
0.5	0.495 ± 0.034	6.87	99.00
50.0	45.800 ± 1.528	3.34	91.60

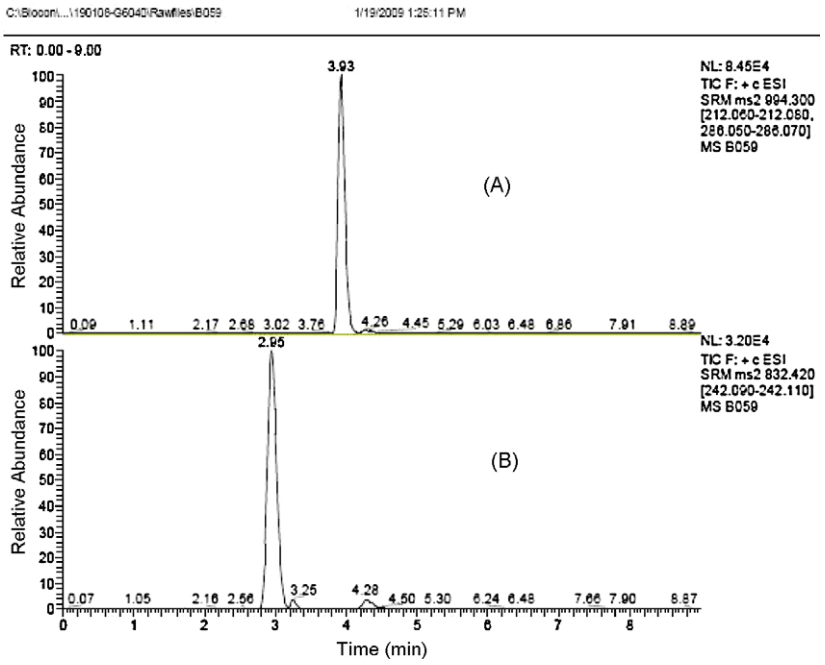

Fig. 7. MRM ion chromatograms of a study sample after administration of 0.5 mg/kg dose of atosiban (A) in presence of 5 μg/ml of eptifibatide (B).

(S/N: 669) at 0.01 μg/ml for atosiban in rat plasma, the assay was found be sensitive and robust. Representative MRM chromatogram of atosiban at 0.01 μg/ml spiked and extracted from rat plasma is given (Fig. 6) in comparison with blank rat plasma (Fig. 5). Good on-column sensitivity was observed for 5 μl injection volume corresponding to 0.0166 ng.

3.1.4. Precision and accuracy

Precision and accuracy of the method were determined by analysing quality control samples (QCs) consisting on rat plasma samples spiked at five different concentrations (Table 1). The between-batch precision and accuracy was determined by six set of quality control samples on two different batches. The quality control samples were processed and analysed immediately following the standard calibration curve. The acceptance criteria of within and between-batch precision was <±15% and accuracy was <15% across the calibration range. The coefficient of variance at LLOQ within-batch was 8.94% and accuracy was 100.0%. The between-batch precision was 8.17% and accuracy was 100.8% for atosiban.

3.1.5. Short term room temperature stability

Bench top stability of atosiban was performed to evaluate the stability in matrix for the time needed to extract the samples. Stability of the QCs was determined at two concentrations (0.5 and 50.0 μg/ml) in five replicates (Table 2). The QCs were kept at room temperature for at least 4 h before extraction and IS was added after intended time period. The results were compared to the nominal concentrations and atosiban was found to be stable in rat plasma at room temperature for at least 4 h.

3.1.6. Freeze/thaw stability

Freeze/thaw stability was performed to evaluate atosiban stability after three freeze/thaw cycles. QCs at two concentrations (0.5 and 50.0 μg/ml) in five replicates were frozen at −85 °C (for minimum of 12 h) and thawed at room temperature. The freeze/thaw cycle was repeated three times. After the completion of the third cycle, the QCs were analysed by adding IS before extraction. Results shown in Table 2 indicate the stability of atosiban after three freeze/thaw cycles.

3.1.7. Autosampler stability

Stability of samples in autosampler was performed to ensure that the quality and integrity of data. QCs at two concentrations (0.5 and 50.0 μg/ml) in five replicates were stored in reconstitution solvent at 4 °C for 72 h prior to injection. A comparison of QCs to the nominal concentration given in Table 2 shows atosiban was stable in reconstitution solvent for at least 72 h.

3.1.8. Long-term frozen-storage stability

The long-term frozen-storage stability of atosiban in rat plasma at −85 °C was evaluated for thirty days in five replicates. The precision and accuracy of atosiban at 0.5 and 50.0 μg/ml after thirty days at −85 °C was 6.87%, 99% and 3.34%, 91.60%, respectively.

3.1.9. Dilution integrity

To assess the dilution integrity (1:2) samples were prepared by spiking the plasma with atosiban at a concentration of 60 μg/ml. Replicate samples were prepared by further diluting these samples to two times with blank rat plasma and processed. The precision and accuracy for dilution integrity (1:2) were determined by measuring the concentrations of diluted samples against freshly prepared calibration standard. The precision and accuracy for 1:2 dilution integrity was found to be 5.03% and 99.7%, respectively.

3.1.10. Summary of validation

The LLOQ in rat plasma was 0.01 μg/ml when 100 μl plasma used. The extraction recovery of atosiban at 0.1 and 50.0 μg/ml concentrations showed average recovery 71.0%. The average extraction recovery at 5.0 μg/ml for IS was 99.5%. Standard calibration curve was generated for atosiban in rat plasma for the concentration range 0.01, 0.05, 0.1, 0.5, 1.0, 5.0, 10, 20.0 and 50.0 μg/ml and the correlation coefficient was 0.9966.

3.2. Kinetic analysis

Representative mass chromatogram of study sample after administration of 0.5 mg/kg dose of atosiban with IS is given in Fig. 7. Peak atosiban concentrations were observed at 2 min post-dose, the first sampling time, in all the treatment groups on Day

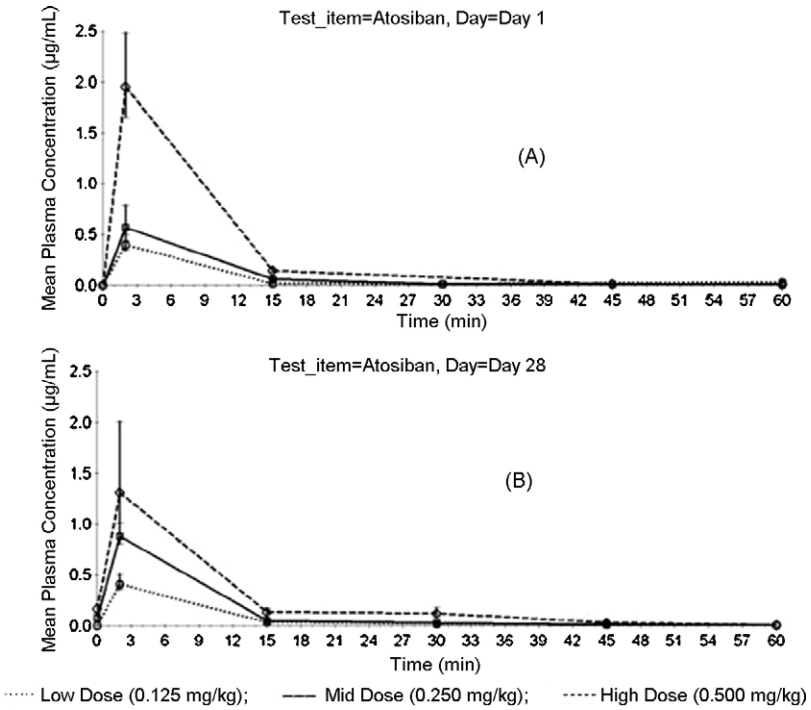

Fig. 8. Mean atosiban plasma concentration vs. time profile in wistar rats following intravenous administration of atosiban on Day 1 (A) and Day 28 (B) (error bars calculated on the basis of mean ± SD).

Table 3
Kinetic parameters of atosiban on Day 1 and Day 28.

Day	Dosage (mg/kg)	Ratio	Atosiban			
			C_{max} (μg/ml)	Ratio	AUC_{last} (min μg/ml)	Ratio
Day 1	0.125		0.40		2.30	
	0.250	2	0.57	1.43[a]	4.29	1.87[a]
	0.500	2	1.95	3.42[b]	12.6	2.94[b]
Day 28	0.125		0.41		3.13	
	0.250	2	0.88	2.15[a]	5.76	1.84[a]
	0.500	2	1.31	1.49[b]	11.34	1.97[b]

AUC: area under curve; C_{max}: concentration maximum, AUC_{last}; area under the curve from time 0–time *t*.
[a] Mid dose (0.250 mg/kg):low dose (0.125 mg/kg) exposure ratio.
[b] High dose (0.500 mg/kg):mid dose (0.250 mg/kg) exposure ratio.

1 and Day 28 (Fig. 8). Mean peak atosiban concentration (C_{max}) was 0.40, 0.57, 1.95 μg/ml on Day 1 and was 0.41, 0.88, 1.31 μg/ml on Day 28 for low, mid and high dose treated animals, respectively (Table 3). The mean AUC_{last} for low, mid and high dose groups were 2.30, 4.29, 12.60 min μg/ml and 3.13, 5.76, 11.34 min μg/ml on Day 1 and Day 28, respectively. The increase in dosage from 0.125 to 0.500 mg/kg resulted in approximately proportionate increase in mean C_{max} on Day 1 and Day 28. A 2-fold increase in dosage from 0.125 to 0.250 mg/kg resulted in 1.43- and 2.15-fold rise in mean C_{max} on Day 1 and Day 28, respectively. A further 2-fold increase in dosage from 0.250 to 0.500 mg/kg resulted in 3.42 and 1.49-fold rise in mean C_{max} on Day 1 and Day 28 of treatment. A 2-fold increase in dose from 0.125 to 0.250 mg/kg resulted in 1.87-fold rise in AUC_{last} on Day 1 and 1.84-fold rise in exposure on Day 28. A further 2-fold increase in dose resulted in 2.94-fold and 1.97-fold rise in AUC_{last} on Day 1 and Day 28 of treatment, respectively. The concentrations of the samples from vehicle control were below quantification limit. The observed increase in the exposure of atosiban treated groups generally approximated dose proportionality. The exposure on Day 1 and Day 28 show no significant accumulation of the atosiban on repeated administration for 28 consecutive days.

4. Conclusions

A novel method for bioanalysis of a cyclic peptide atosiban is reported. The method is sensitive to quantify atosiban down to 0.01 μg/ml from 100 μl rat plasma. The reported method showed on-column sensitivity of 0.0166 ng for 5 μl injection volume. Extraction method showed average recovery of 71% for atosiban and 99.5% for IS. Standard calibration curve generated for atosiban at 0.01, 0.05, 0.1, 0.5, 1.0, 5.0, 10, 20.0 and 50.0 μg/ml with 5 μg/ml of IS showed correlation coefficient 0.9966. Specificity experiments confirmed no significant plasma protein interference for atosiban or IS. The between-batch precision was 8.17% and accuracy was 106.15%. The within-batch precision was 8.94% and accuracy was 114.0% for atosiban. The precision for dilution integrity was found to be 5.03% for 1:2 dilutions and accuracy was 99.7%. Various stability parameters such as short term room temperature stability, freeze/thaw stability, autosampler stability and long-term frozen-

storage stability performed on atosiban confirmed suitability of the method for bioanalysis.

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