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Quantification of cinacalcet by LC–MS/MS using liquid–liquid extraction from 50 μL of plasma

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ABSTRACT

A simple and economical high-performance liquid chromatography–positive ion electrospray tandem mass spectrometry method was developed and validated for the quantification of cinacalcet in plasma. Following liquid–liquid extraction, the analyte was separated using an isocratic mobile phase on a reversed-phase column and analyzed by MS/MS in the multiple reaction monitoring mode using the respective $[M+H]^+$ ions, *m/z* 358–155 for cinacalcet and *m/z* 310–148 for the internal standard. The assay exhibited a linear dynamic range of 0.1–200 ng/mL for cinacalcet in plasma. Acceptable precision (<10%) and accuracy (100 ± 5%) were obtained for concentrations over the standard curve range. A run time of 3.5 min for each sample made it possible to analyze more than 250 samples per day. The method was successfully applied to quantify cinacalcet concentrations in a preclinical pharmacokinetic study after a single oral administration of cinacalcet at 10 mg/kg to rats. Following oral administration the maximum mean concentration in plasma (C_{max}; 160 ± 56 ng/mL) was achieved at 1.0 h (T_{max}), area under the curve (AUC) and half-life ($t_{1/2}$) were 949 ± 257 ng h/mL and 3.58 ± 0.4 h, respectively.

1. Introduction

Cinacalcet, the only commercially available calcimimetic, is indicated for the treatment of secondary hyperparathyroidism in patients with chronic kidney disease receiving dialysis and for the treatment of hypercalcaemia in patients with parathyroid carcinoma [1]. Cinacalcet, a member of the phenylethylamine type II calcimimetic family, acts at the calcium-sensing receptor in the parathyroid gland to decrease secretion of parathyroid hormone [2].

Following oral administration peak plasma concentrations of cinacalcet occur within 2–6 h. The absolute bioavailability is about 20–25%, and administration of cinacalcet with low- and high-fat meals increases exposure 1.5- to 1.8-fold [3]. Cinacalcet is eliminated mainly through metabolism. The circulating metabolites of cinacalcet are inactive, and <1% of the parent drug is excreted in the urine [4]. The pharmacokinetics of cinacalcet is dose proportional over the dose range of 30–180 mg and are not altered by the extent of renal impairment or the method of dialysis [5,6]. After administration of cinacalcet 50 mg/day, C_{max} was 18.6 and 20.2 ng/mL, respectively, in patients with primary hyperparathyroidism and with secondary hyperparathyroidism at steady state [1]. The terminal elimination half life is 30–40 h and steady state concentrations are achieved before 7 days [1].

The bioanalytical component of a pharmacokinetic study requires a drug assay with simplicity, selectivity, sensitivity, small volume requirements, and rapid turnaround time. Most of the liquid chromatography tandem mass spectrometry (LC–MS/MS) methods [4,7,8] used reversed-phase chromatography except Padhi et al. [9] method in which normal phase chromatography was used for determining cinacalcet concentrations in human plasma samples. In these LC–MS/MS methods, the cinacalcet was extracted from plasma samples using solid phase extraction (SPE) technique [4,7,8] and heptadeuterated cinacalcet was used as an internal standard [4] and also the plasma volume requirement of 500 μL was very high [8]. However, all these methods have sensitivity of 0.1 ng/mL which is sufficient enough for the pharmacokinetic studies of cinacalcet.

The present method consisted of a liquid–liquid extraction of cinacalcet and fluoxetine (commercially available internal standard) from lower plasma sample volumes (50 μL) with diethyl ether–dichloromethane (70:30, v/v). After extraction, the samples were injected onto a Waters Symmetry C_{18} reversed-phase chromatographic column for separation. The analyte was detected by tandem mass spectrometry using positive electrospray ionization in multiple reaction monitoring (MRM) mode. The run time of the present method is 3.5 min, which insures high throughput. The method was validated over the concentration range of 0.1–200 ng/mL.

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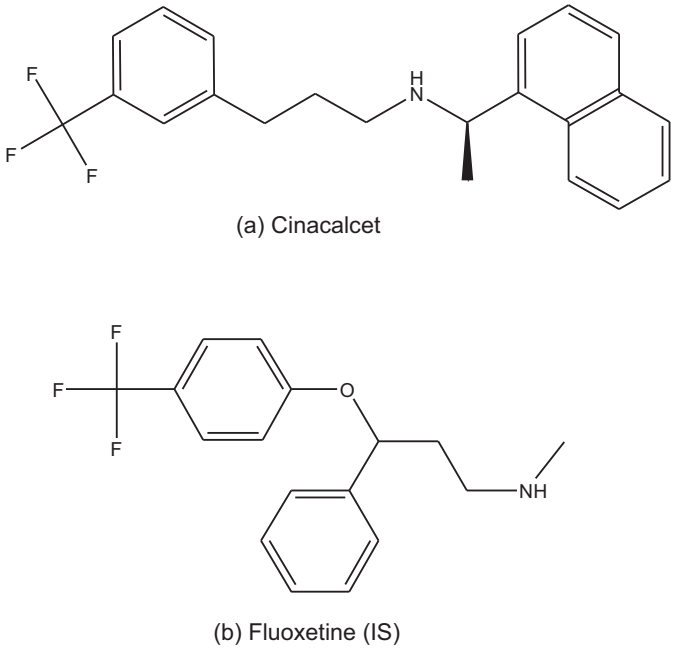


Fig. 1. Chemical structures for cinacalcet and the IS (fluoxetine).

This paper describes a simple, economical, selective, rapid and reproducible triple quad mass spectrometric method with commercially available internal standard for the quantification of cinacalcet in rat plasma.

2. Experimental

2.1. Chemicals

Cinacalcet hydrochloride and fluoxetine hydrochloride (internal standard) drug substances were obtained from R&D department of Suven Life Sciences Ltd. (Hyderabad, India). Chemical structures are presented in Fig. 1. HPLC-grade LiChrosolv methanol and HPLC-grade LiChrosolv acetonitrile were purchased from Merck (Darmstadt, Germany). Ammonium acetate, formic acid, diethyl ether, dichloromethane and ortho phosphoric acid were purchased from Merck (Worli, Mumbai, India). HPLC grade water from Milli-Q system (Millipore, Bedford, MA, USA) was used. All other chemicals were of analytical grade.

2.2. LC–MS/MS instrument and conditions

The HPLC SIL HTC system (Shimadzu Corporation, Kyoto, Japan) is equipped with LC-AD VP binary pump, a DGU20A5 degasser and a SIL-HTC auto sampler equipped with a CTO-10AS VP thermostated column oven. The chromatography was performed using column Waters Symmetry® C18, 4.6 × 100 mm, 3.5 μm (Waters Corporation, Dublin, Ireland) at a temperature of 30 °C. The isocratic mobile phase composition was a mixture of 10 mM ammonium acetate adjusted to pH 4.0 with diluted formic acid and acetonitrile (5:95, v/v), which was pumped at a flow-rate of 1.0 mL/min with split ratio of load to waste 10:90.

Mass spectrometric detection was performed on an API 3000 triple quadrupole instrument (MDS-SCIEX, Concord, Ontario, Canada) using multiple reaction monitoring (MRM). A turboionspray interface operating in positive ionization mode was used. Typical source conditions were as follows: the turbo-gas temperature was set at 250 °C, and the ion spray needle voltage was adjusted at 3500 V. The mass spectrometer was operated at unit resolution for both Q1 and Q3 in the MRM mode, with a dwell time of 200 ms per MRM channel. The precursor/product ion pairs monitored were m/z 358–155 for cinacalcet and m/z 310–148 for the internal standard (IS). Nebulizer Gas was set at 4 (arbitrary units); curtain gas was set at 6 (arbitrary units) and the collision gas was set at 5 (arbitrary units). The collision energy was set at 25 and 13 V for cinacalcet and for the IS, respectively. Data acquisition was performed with analyst 1.4.2 software (MDS-SCIEX, Concord, Ontario, Canada).

2.3. Sample preparation

Standard stock solutions of cinacalcet (1 mg/mL) and the IS (1 mg/mL) were separately prepared in methanol. Working solutions for calibration and controls were prepared by appropriate dilution in water–methanol (50:50, v/v; diluent). The IS working solution (10 μg/mL) was prepared daily by diluting its stock solution with diluent. Working solutions (0.5 mL) were added to drug-free rat plasma (9.5 mL) as a bulk, to obtain cinacalcet concentration levels of 0.1, 0.2, 0.4, 1, 10, 20, 40, 100 and 200 ng/mL as a single batch at each concentration. Quality control (QC) samples were also prepared as a bulk on an independent weighing of standard drug, at concentrations of 0.1 (LLOQ), 0.3 (low), 80 (medium) and 160 ng/mL (high) as a single batch at each concentration. The calibration and quality control bulk samples were divided into aliquots in micro centrifuge tubes (Axygen, INC., Union City, CA, USA; 0.6 mL) and stored in the freezer at below −50 °C until analysis.

A plasma sample (50 μL) was pipetted into a 15 mL glass tube and then 5 μL of the IS working solution (10.0 μg/mL) was added. After vortex mixing for 10 s, 10 μL of 10% ortho phosphoric acid was added. After vortex mixing for 10 s, 2.5 mL aliquot of the extraction solvent, diethyl ether:dichloromethane (70:30, v/v), was added and the sample was vortex-mixed for 3 min. The organic layer (2.0 mL) was transferred to a glass tube and evaporated to dryness using an evaporator at 40 °C under a stream of nitrogen. Then the dried extract was reconstituted in 250 μL of reconstitution solvent (10 mM ammonium acetate adjusted to pH 4.0 with diluted formic acid and acetonitrile, 5/95, v/v) and a 10-μL aliquot was injected into the chromatographic system.

2.4. Bioanalytical method validation

A calibration curve was constructed from a blank sample (a plasma sample processed without the IS), a zero sample (a plasma sample processed with the IS) and nine non-zero samples covering the total range of 0.1–200 ng/mL, including the LLOQ. The calibration curves were generated using the analyte to the IS peak area ratios by weighted ($1/x^2$) least-squares linear regression on consecutive days. The acceptance criterion for a calibration curve was a correlation coefficient (r) of 0.99 or better, and that each back-calculated standard concentration must be within 15% deviation from the nominal value except at the LLOQ, for which the maximum acceptable deviation was set at 20%. At least 67% of non-zero standards were required to meet the above criteria, including acceptable LLOQ and upper limit of quantification.

The within-batch precision and accuracy were determined by analyzing four sets of QC samples (LLOQ, low, medium and high concentrations) each comprised of six replicates in a batch. The between-batch precision and accuracy were determined by analyzing such five different batches. The acceptance criteria for within- and between-batch precision were 20% or better for LLOQ and 15% or better for the other concentrations, and the accuracy were 100 ± 20% or better for LLOQ and 100 ± 15% or better for the other concentrations.

Recovery of cinacalcet from the extraction procedure was determined by a comparison of the peak area of cinacalcet in spiked

plasma samples (six each of low, medium and high QCs) with the peak area of cinacalcet in samples prepared by spiking extracted drug-free plasma samples with the same amounts of cinacalcet at the step immediately prior to chromatography. Similarly, recovery of the IS was determined by comparing the mean peak areas of extracted medium QC samples ($n = 6$) to mean peak areas of the IS in samples prepared by spiking extracted drug-free plasma samples with the same amounts of the IS at the step immediately prior to chromatography.

The stability of the analyte and the IS in rat plasma under different temperature and timing conditions, as well as their stability in the stock solutions were evaluated. QC samples were subjected to short-term room temperature conditions, long-term storage conditions (−50 °C) and freeze-thaw stability studies. All the stability studies were conducted at two concentration levels (0.3 and 160 ng/mL as low and high values) with six replicates for each. All these stability samples were compared against freshly prepared samples.

2.5. Pharmacokinetic analysis

The WinNonlin version of 5.3 (Pharsight Corporation, Mountain View, Canada) was used for the pharmacokinetic analysis. Plasma concentration time profile for cinacalcet was analyzed by noncompartmental methods and individual pharmacokinetic parameters for each rat were calculated. After dosing, the maximum plasma concentration (C_{max}) and the time to reach maximum plasma concentration (T_{max}) were recorded. The terminal elimination rate constant (K_e) was determined by regression analysis of the log linear terminal segment of the plasma concentration–time curve and the terminal half-life ($t_{1/2}$) was calculated as $t_{1/2} = \ln 2/K_e$. The area under the plasma concentration–time curve (AUC) was calculated according to the trapezoidal rule extrapolating to infinity.

3. Results and discussion

3.1. Mass spectrometry

In order to develop a method with the desired LLOQ (0.1 ng/mL), it was necessary to use MS–MS detection, as MS–MS methods provide improved limit of detection and selectivity. The inherent selectivity of MS–MS detection was also expected to be beneficial in developing a selective and sensitive method. $[M+H]^+$ was the predominant ion in the Q1 spectrum and was used as the precursor ion to obtain product ion spectra. The product ion mass spectra, and their proposed rationalizations in terms of fragmentation patterns of cinacalcet and the IS are illustrated in Fig. 2. The product ion mass spectrum of cinacalcet showed predominant fragment ions at m/z 204 and 155. Cinacalcet in the positive ionization mode shows a selective α-cleavage next to the aliphatic nitrogen, resulting in the product ion m/z 155 and m/z 204 (Fig. 2A). The product ion mass spectrum of the IS showed the formation of characteristic product ions at m/z 148 and 44. Fragmentation of the IS in the positive ionization mode shows the loss of 4-trifluoromethyl-phenol group resulting in the product ion m/z 148 (Fig. 2B). The most sensitive mass transition was from m/z 358–155 for cinacalcet and m/z 310–148 for the IS.

3.2. Optimization of extraction procedures

Liquid–liquid extraction (LLE) was used for the sample preparation in this work. Six organic solvents, n-hexane, ethyl acetate, diethyl ether, dichloromethane, chloroform, tert-butyl methyl ether and their mixtures in different combinations and ratios were evaluated. Finally combination of diethyl ether and dichloromethane (70:30 v/v) was found to be optimal, which can produce a clean chromatogram for a blank plasma sample and consistent recovery for the analyte. The average extraction procedure recovery of cinacalcet from spiked plasma samples was 86.2 ± 4.1% and of the IS was 84.7 ± 1.7% at the concentration used in the assay (10 μg/mL). Both the extraction procedure recoveries of the analyte and the IS were good and it was consistent, precise and reproducible. The assay has proved to be robust in high-throughput bioanalysis.

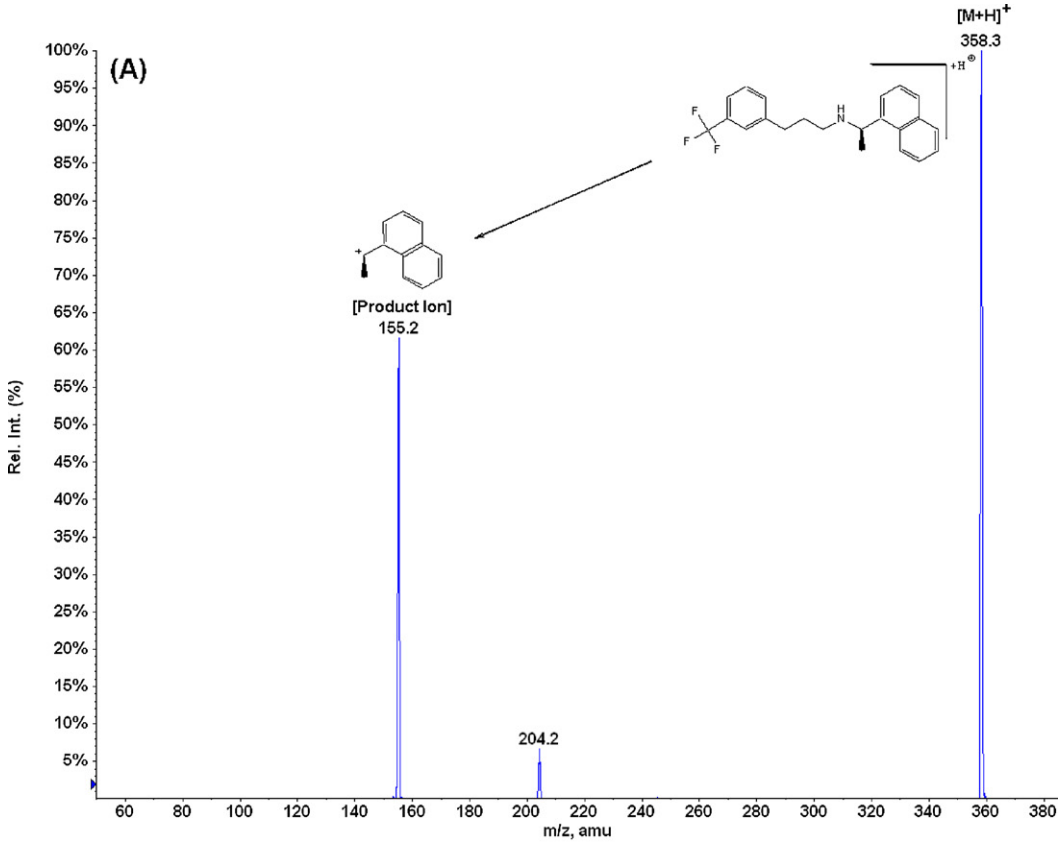
A good internal standard should mimic the analyte in the entire sample extraction, chromatographic elution and mass spectrometric detection. It should track the analyte during the extraction and compensate for any potential recovery inconsistency. It will elute together with the analyte on the column and compensate for any potential inconsistent response due to matrix effects. Stable isotopically labeled internal standards are ideal candidates for meeting the above criteria. However, isotopes are not always easily accessible due to the prohibitive high cost or due to the technical difficulty in synthesizing them. Several compounds were investigated to find a suitable IS, and finally fluoxetine, commercially available compound, was found to be suitable which has similar structural and chromatographic properties to cinacalcet (Fig. 1). Clean chromatograms were obtained and no significant direct interferences in the MRM channels at the relevant retention times were observed. This approach of IS selection would likely be adequate for preclinical species, but a stable labeled IS would be highly desirable for clinical applications given the patient populations in which this drug is used, and as some of these patients may be seriously ill and receiving multiple co-medications. All validation experiments in this assay were performed with matrixes obtained from pooled rat samples. As all data fall within 15%, it can be concluded that the degree of matrix effect was sufficiently low to produce acceptable data, and the method can be considered as valid.

3.3. Optimization of chromatographic conditions

Several different chromatographic columns, including Zorbax XDB®, YMC-Pack ODS-AQ®, Waters Atlantis® C18, Chromolith Performance®, and Waters Symmetry® C18 were tested to optimize the good peak shape and response. Series of experiments were conducted to select the best stationary and mobile phases that would give optimum peak shape and response. A significantly good peak shape and response were obtained using Waters Symmetry C18, 4.6 mm × 100 mm, 3.5 μm column.

The chromatographic conditions, especially the composition of mobile phase, were optimized through several attempts to achieve good resolution and symmetric peak shapes for the analyte and the IS, as well as a short run time. It was found that a mixture of 10 mM ammonium acetate buffer (pH adjusted to 4.0 with formic acid) and acetonitrile (5:95, v/v) could achieve this purpose and was finally adopted as the mobile phase. The high proportion of organic solvent eluted the analyte and the IS at the retention time of 2.30 min and 1.95 min, respectively. A flow rate of 1.0 mL/min produced good peak shapes and permitted a run time of 3.5 min.

The pH of the aqueous phase of the liquid chromatographic mobile phase influences both the chromatographic elution of the compounds and the formation of the $[M+H]^+$ molecular ions and is strongly related to their degree of ionization. The pKa values of the analyte and the IS were calculated using the MarvinSketch/Swing 4.0.3 software. As both cinacalcet and fluoxetine are basic compounds with pKa values 10.3 and 9.8, respectively, the use of slightly acidic solutions favors ionization of the analytes by protonation of their basic sites. Therefore, it was found that, positive ionization of the compounds in the electrospray ion source increases in acidic mobile phases. Hence the pH of ammonium acetate buffer was adjusted to 4.0 with formic acid to obtain high sensitivity and good peak shapes.



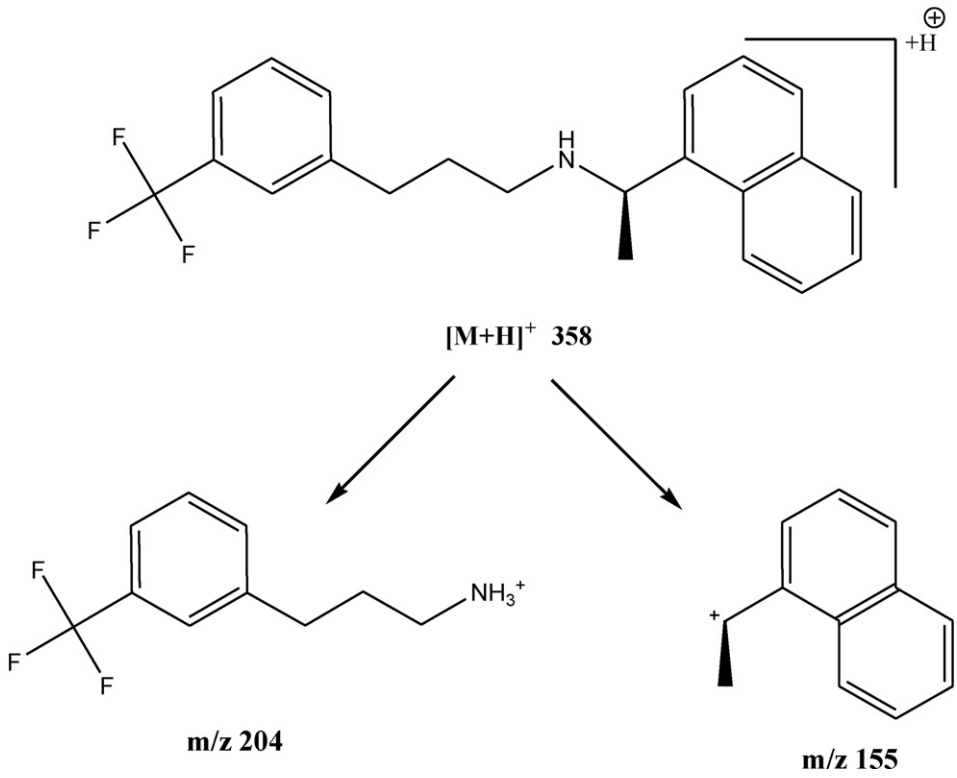


Fig. 2. Full scan positive ion turbo ion spray product ion mass spectra and the proposed patterns of fragmentation of (a) cinacalcet and (b) fluoxetine (internal standard).

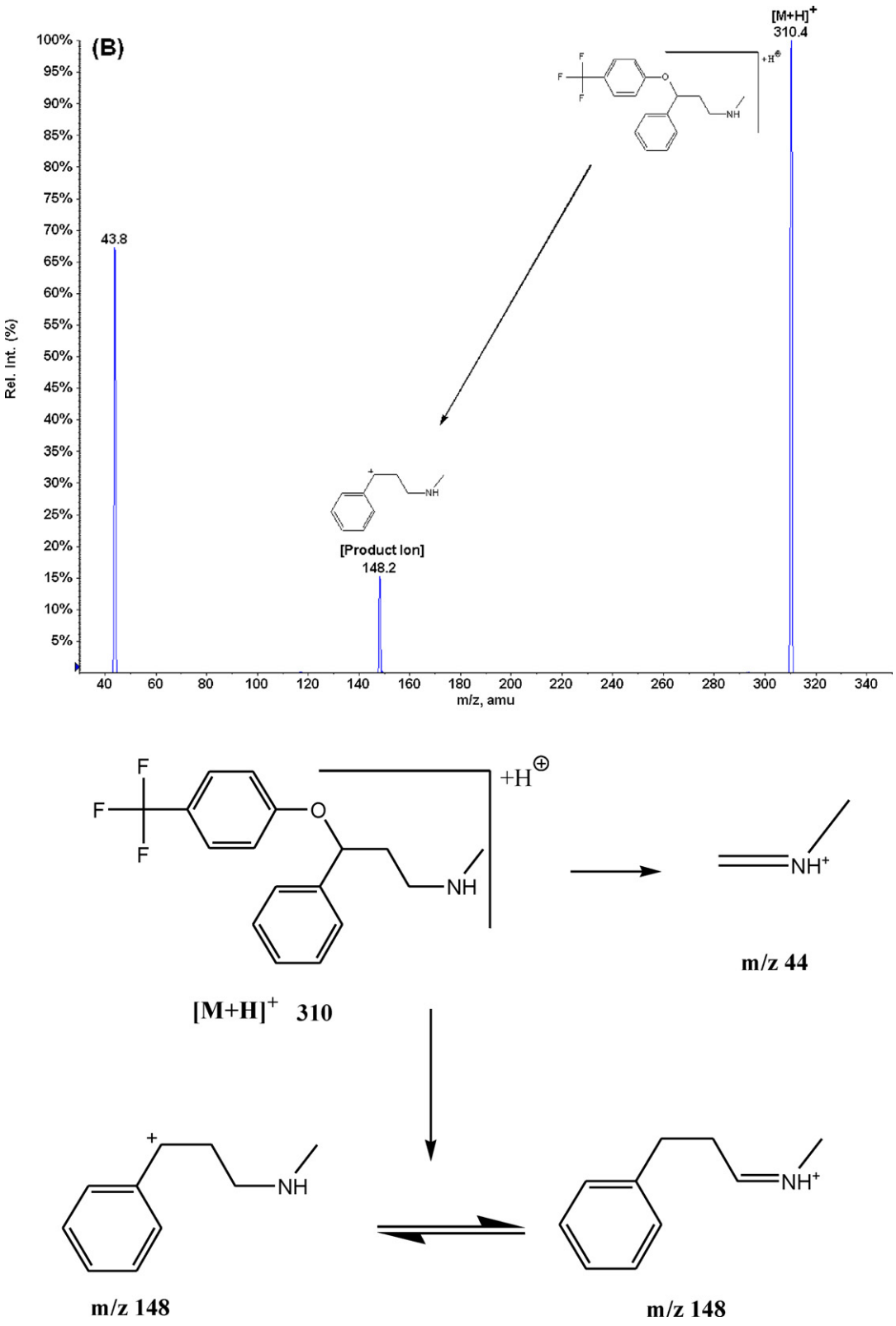


Fig. 2. (*contineud*)

3.4. Assay performance and validation

The nine-point calibration curve was linear over the concentration range of 0.1–200 ng/mL. The calibration model was selected based on the analysis of the data by linear regression with/without intercepts and weighting factors ($1/x$, $1/x^2$ and none). The best linear fit and least-squares residuals for the calibration curve were achieved with a $1/x^2$ weighing factor, giving a mean linear regression equation for the calibration curve of:

$$y = 0.0325(\pm 0.0126)x + 0.0006(\pm 0.0007)$$

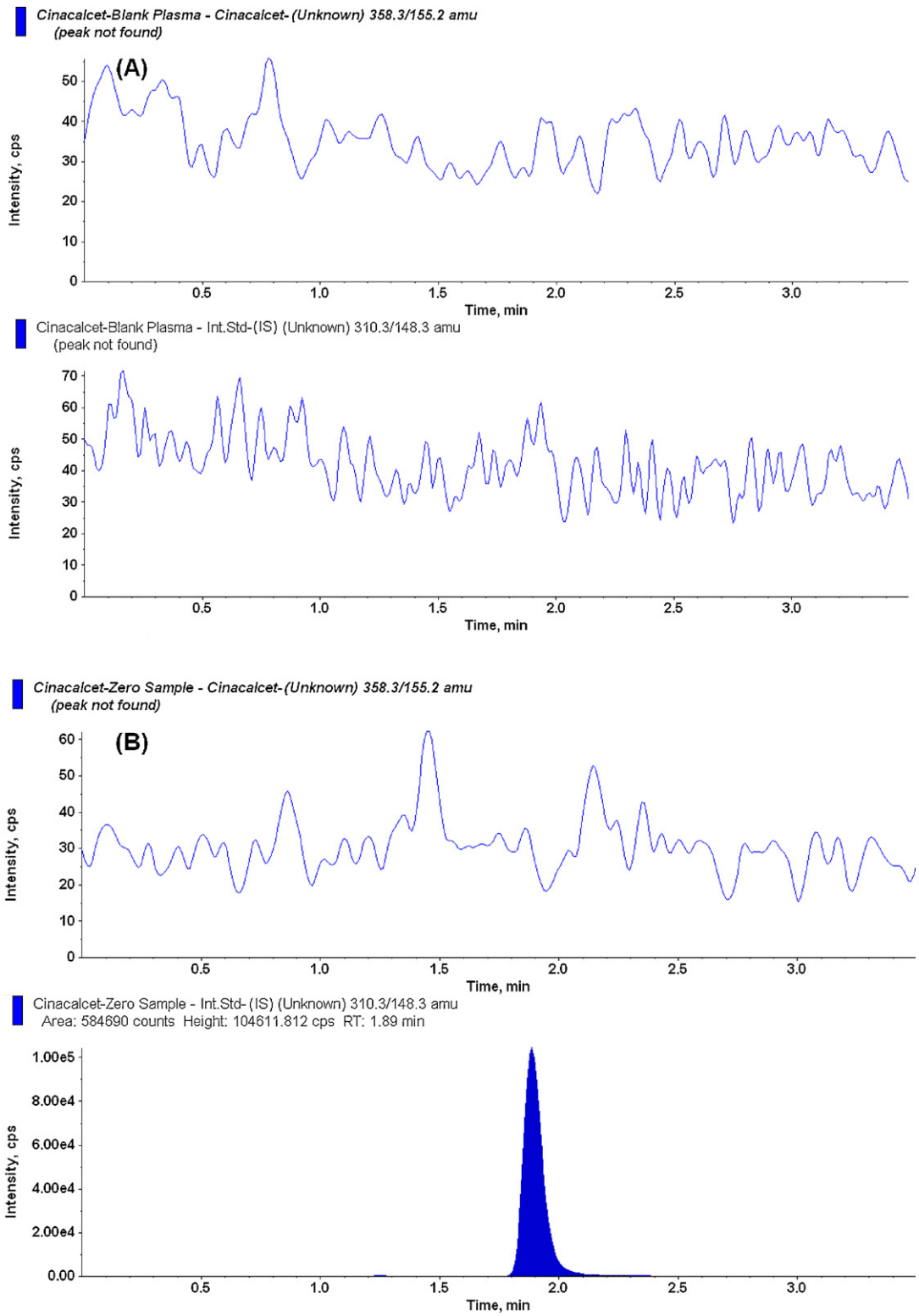


Fig. 3. MRM chromatograms in rat plasma for cinacalcet and the IS resulting from analysis of (a) blank (drug and the IS free) rat plasma (b) zero sample (drug-free spiked with the IS) rat plasma and (c) 0.1 ng/mL (LLOQ) of cinacalcet spiked with the IS.

where y was the peak area ratio of the analyte to the IS and x was the concentration of the analyte. The mean correlation coefficient of the weighted calibration curve generated during the validation was 0.9983 ± 0.0002.

The selectivity of the method was examined by analyzing ($n = 6$) blank rat plasma extract (Fig. 3A) and an extract spiked only with the IS (Fig. 3B). As shown in Fig. 3A, no significant direct interference in the blank plasma traces was observed from endogenous substances in drug-free rat plasma at the retention time of the analyte. Similarly, Fig. 3B shows the absence of direct interference from the IS to the MRM channel of the analyte. Fig. 3C depicts a representative ion-chromatogram for the LLOQ (0.1 ng/mL) in

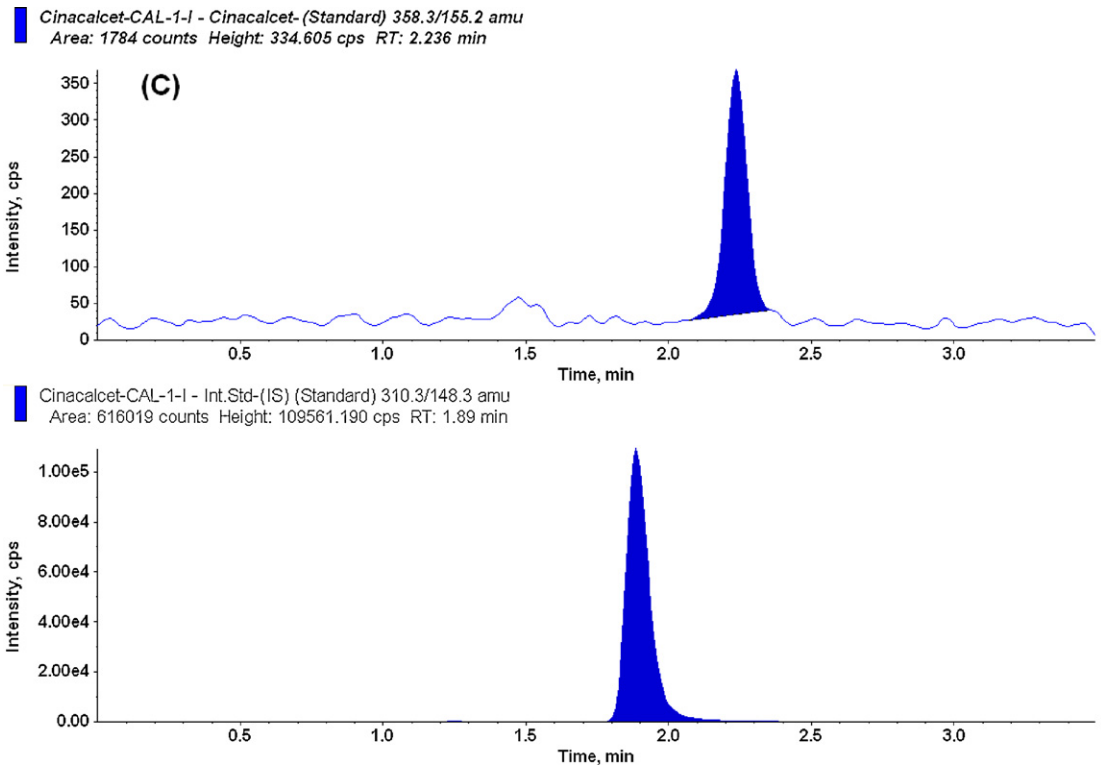


Fig. 3. (*contineud*)

rat plasma. Excellent sensitivity was observed for a 10-μL injection volume; the LLOQ corresponds to ca. 0.17 pg on-column. The mean response for the analyte peak at the assay sensitivity limit (0.1 ng/mL) was ≈10-fold greater than the mean response for the peak in six blank rat plasma samples at the retention time of the analyte. The between-batch precision at the LLOQ was 9.9%, and the between-batch accuracy was 103.7% (Table 1). The within-batch precision was 9.8% and the accuracy was 101.1% for cinacalcet.

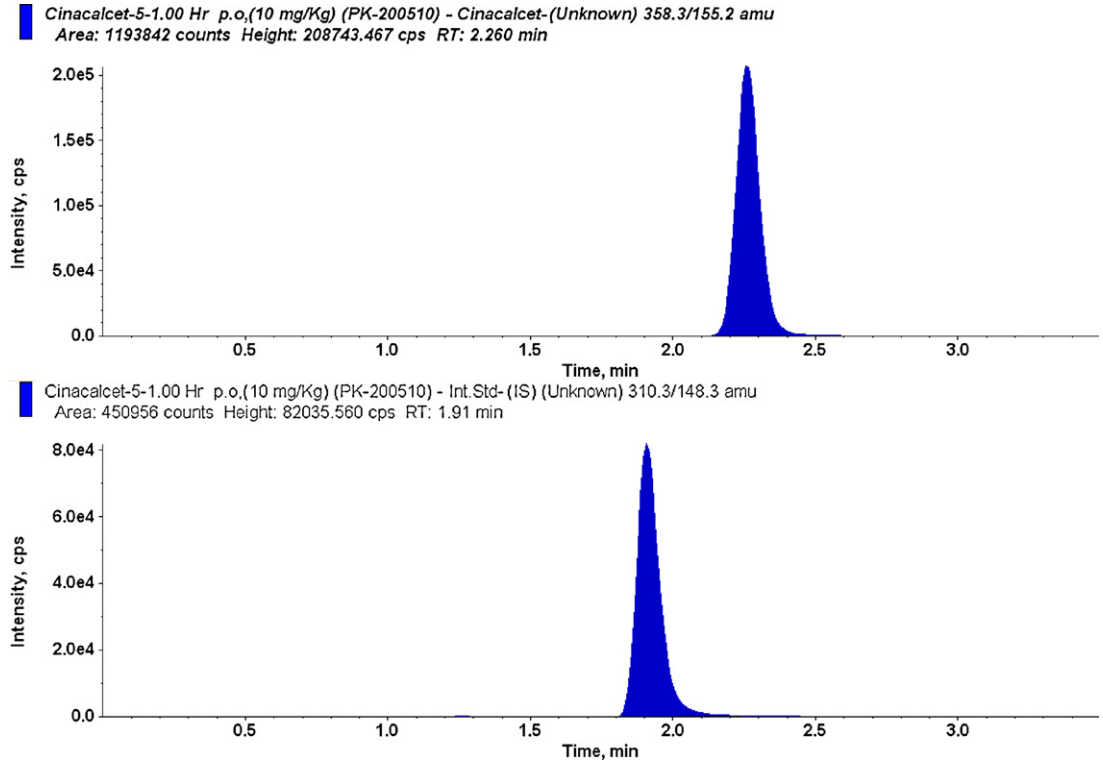


Fig. 4. Representative MRM chromatograms resulting from the analysis of 1 h post dose plasma sample after the oral administration of cinacalcet (10 mg/kg) to rat shows 185 ng/mL concentration.

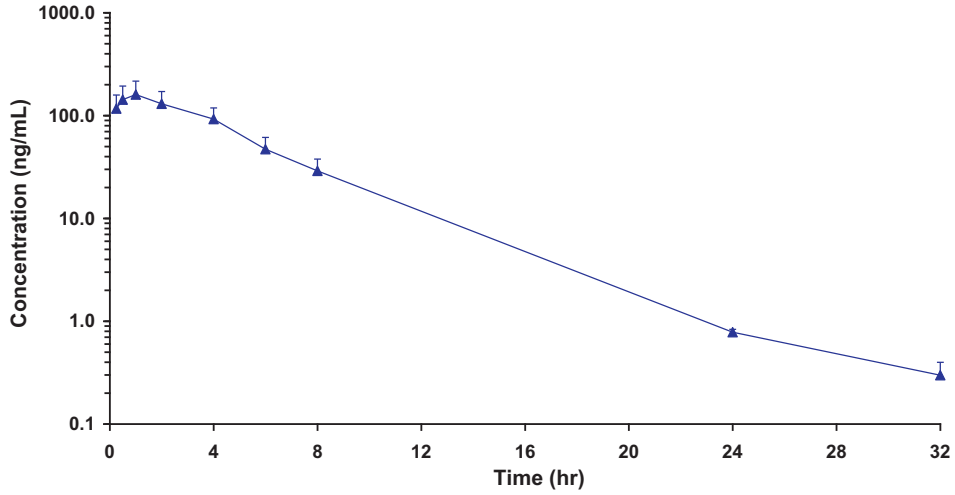


Fig. 5. Plasma concentration vs time profiles of cinacalcet after single dose oral administration of 10 mg/kg in male rats. The data points are means and standard deviation bars of three observations.

Table 1
Precision and accuracy of the method for determining cinacalcet concentrations in rat plasma samples.

Concentration added (ng/mL)	Between-batch ($n = 30$)			Within-batch ($n = 12$)		
	Concentration found (mean ± S.D.) (ng/mL)	Precision (%)	Accuracy (%)	Concentration found (mean ± S.D.) (ng/mL)	Precision (%)	Accuracy (%)
0.1	0.10 ± 0.01	9.9	103.7	0.10 ± 0.01	9.8	101.1
0.3	0.31 ± 0.02	5.8	104.2	0.31 ± 0.02	5.0	102.3
80	77.09 ± 2.73	3.5	96.4	77.95 ± 2.47	3.2	97.4
160	158.84 ± 7.04	4.4	99.3	157.65 ± 6.25	4.0	98.5

The lower and upper quantification levels of cinacalcet ranged from 0.3 to 160 ng/mL in rat plasma. For the between-batch experiments the precision ranged from 3.5 to 5.8% and the accuracy from 96.4 to 104.2% (Table 1). For the within-batch experiments the precision and accuracy for the analyte met the acceptance criteria.

3.5. Stability studies

For short-term stability determination, stored plasma aliquots were thawed and kept at room temperature for a period of time exceeding that expected to be encountered during routine sample preparation (around 24 h). Samples were extracted and analyzed as described above and the results indicate reliable stability behavior under the experimental conditions of the regular analytical procedure. The stability of QC samples kept in the autosampler for 26 h was also assessed. The results indicate that solutions of the analyte and the IS can remain in the autosampler for at least 26 h without showing significant loss in the quantified values, indicating that samples should be processed within this period of time.

The stability data of the analyte in plasma over three freeze-thaw cycles indicate that the analyte is stable in rat plasma for three freeze-thaw cycles, when stored at below −50 °C and thawed to room temperature.

The long-term stability data of the analyte in rat plasma stored for a period of 36 days at below −50 °C showed reliable stability behavior, as the mean of the results of the tested samples were within the acceptance criteria of ±15% of the initial values of the controls. These findings indicate that storage of the analyte in plasma samples at below −50 °C is adequate, and no stability-related problems would be expected during routine analyses for pharmacokinetic studies.

The stability of the stock solutions was tested and established at room temperature for 8 h, 28 h and under refrigeration (∼4 °C) for 36 days (data not shown). The results revealed optimum stability for the prepared stock solutions throughout the period intended for their daily use.

3.6. Application

To demonstrate the applicability of the LC–MS/MS method, an oral pharmacokinetic study was carried out in male Sprague Dawley rats. The method was used to quantify concentrations of cinacalcet in the plasma of rats, which received oral administration of 10 mg/kg dose of cinacalcet. Plasma samples were frozen at −50 °C until analyzed. The MRM chromatograms obtained for extracted rat plasma sample was depicted in Fig. 4 and the concentration of cinacalcet was 185 ng/mL. The mean plasma concentration time profiles of cinacalcet at 10 mg/kg oral dose were presented in Fig. 5. Inspection of Fig. 5 reveals that the newly developed analytical method has the required sensitivity to characterize the absorption, distribution and elimination phases of cinacalcet following oral dosing. After oral administration the maximum mean concentration in plasma (C_{max}; 160 ± 56 ng/mL) was achieved at 1.0 h (T_{max}) and area under curve (AUC) was 949 ± 257 ng h/mL (Table 2).

Table 2
Calculated pharmacokinetic parameters ($n = 3$).

Pharmacokinetic parameter	(Mean ± S.D.)
AUC (ng h/mL)	949.20 ± 257.30
C_{max} (ng/mL)	160.42 ± 55.92
$t_{1/2}$ (h)	3.58 ± 0.42
T_{max} (h)	1.00 ± 0.00

4. Conclusions

In summary, a method is described for the quantification of cinacalcet in rodent plasma by LC–MS/MS in positive electrospray ionization mode using fluoxetine as internal standard and fully validated according to commonly accepted criteria. The current method has shown acceptable precision and adequate sensitivity for the quantification of cinacalcet in rat plasma samples obtained from rodent pharmacokinetic study. The desired sensitivity of cinacalcet was achieved with an LLOQ of 0.1 ng/mL using the plasma volume of 50 μL. Many variables related to the electrospray reproducibility were optimized for both precision and sensitivity to obtain these results. The simplicity of the assay and using rapid liquid–liquid extraction and sample turnover rate of 3.5 min per sample, make it an attractive procedure in high-throughput bioanalysis of cinacalcet. In adaptability of cinacalcet assay in rat plasma to human plasma for clinical trials, a stable labeled IS would be desirable.

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