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Quantitative determination of odanacatib in human plasma using liquid–liquid extraction followed by liquid chromatography–tandem mass spectrometry analysis

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ABSTRACT

Odanacatib (ODN, MK-0822) is an investigational drug under development for the treatment of osteoporosis. A quantitative LC/MS–MS methodology was developed and validated to determine ODN concentrations in human plasma, with a linear calibration range from 0.500 to 500 ng/mL. Stable isotope $^{13}C_6$-labeled ODN was employed as the internal standard (IS). Sample preparation was based on liquid–liquid extraction of basified plasma with methyl t-butyl ether in a 96-well plate format. The extracted samples were analyzed on a liquid chromatography–tandem mass spectrometry system equipped with a turbo ion spray source. Chromatographic separation of the analyte and IS was achieved on a Phenomenex Luna C18 (50 mm × 2.0 mm, 5 μm) column. Ion pairs m/z 526 → 313 for the analyte and m/z 532 → 319 for the IS were monitored in positive ionization mode for MS detection. This methodology has been fully validated and proved to be rugged and reproducible. Intra- and inter-run variability was within 5.88%, with accuracy between 95.6 and 106% of the nominal concentrations. Analyte stability was evaluated under various sample preparation, analysis and storage conditions. This assay has been utilized to analyze human plasma samples obtained from phase I to III clinical trials.

1. Introduction

Osteoporosis is a worldwide common disease featuring low bone mineral density, which can lead to increased skeletal fragility and greater risk for fracture. It occurs when bone resorption exceeds bone formation during the bone remodeling process [1]. Osteoclasts play a key role in mediating bone resorption, which require an acidic microenvironment and the action of proteases for bone demineralization and organic matrix digestion. Cathepsin K (Cat-K) is the most abundant lysosomal cysteine protease expressed by the osteoclasts that is responsible for the degradation of the organic bone matrix [2]. Reduced bone resorption and increased bone mineral density after selective inhibition of Cat-K have been demonstrated in both preclinical and clinical studies [2]. Based on these findings, Cat-K has been identified as a promising therapeutic target for osteoporosis. Currently, several drug candidates of this class have entered development.

Odanacatib (ODN, MK-0822; Merck, Whitehouse Station, NJ) is a potent and selective inhibitor of Cat-K [3,4]. As a once-weekly, orally administered drug for the treatment of osteoporosis and bone metastasis, ODN has established clinical proof-of-concept [5–7]. The half-life of ODN was between 66 and 93 h for the dosing regimens studied. A Phase I study in post-menopausal women demonstrated significantly increase in bone mineral density (BMD) in a dose-dependent manner, with the greatest reduction observed for weekly 50-mg doses and 2.5 mg daily doses [5]. In a 2-year Phase II study, substantial and progressive increase in BMD was observed in post-menopausal women with low BMD [7]. These studies also showed ODN to be generally well-tolerated. Clinical phase III trials are now underway for ODN.

To study the pharmacokinetic properties of ODN in human subjects, it was necessary to develop an analytical assay to quantitatively measure ODN concentrations in plasma. This assay needed to be highly reproducible and robust; additionally, high throughput was desirable to accelerate the bioanalysis turnaround, given that it was intended to be applied to the analyses of a large number of samples obtained from clinical trials.

Due to its sensitivity, selectivity and flexibility, liquid chromatography coupled with tandem mass spectrometry (LC/MS–MS) has become the tool of choice for small molecule bioanalysis,

Abbreviations: ODN, Odanacatib; Cat K, Cathepsin K; SIL, Stable-isotope labeled; IS, Internal standard; LC, Liquid chromatography; MS, Mass spectrometry; MS–MS, Tandem mass spectrometry; LLE, Liquid–liquid extraction; LLOQ, Lower limit of quantitation; ULOQ, Upper limit of quantitation; QC, Quality control; MTBE, Methyl t-butyl ether; ISR, Incurred sample reproducibility.

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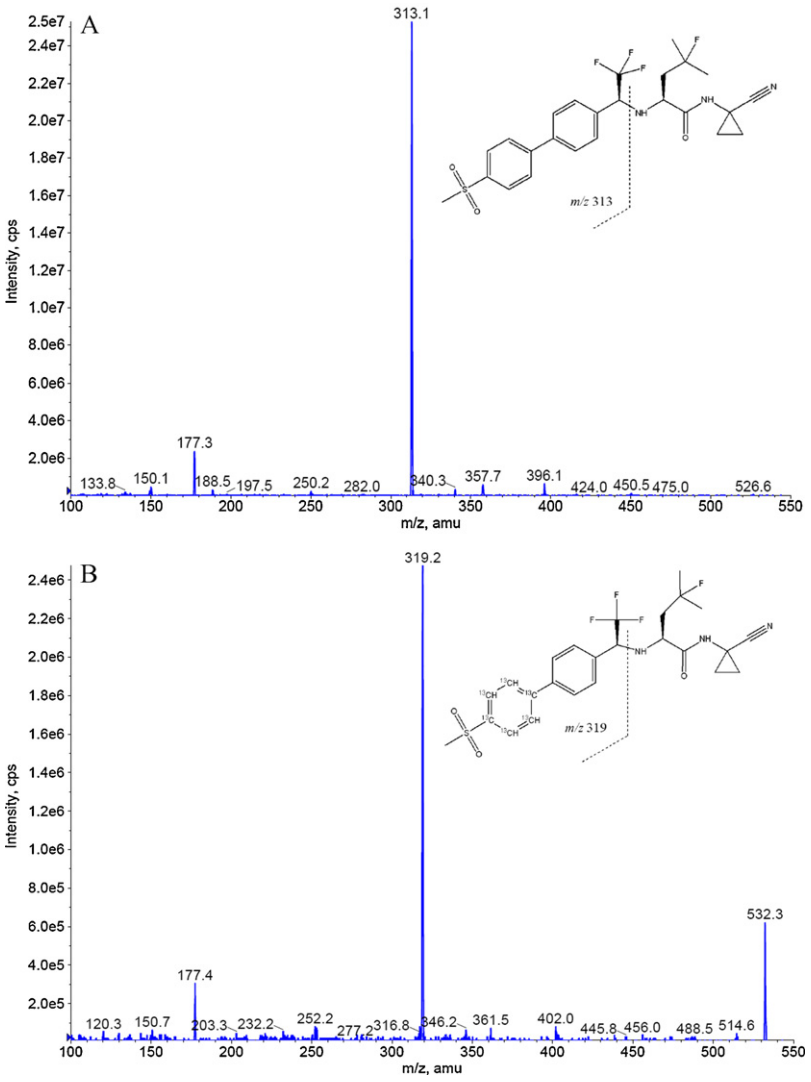

Fig. 1. Product ion mass spectra for (A) ODN and (B) $^{13}C_6$-ODN (IS).

with myriad applications in the analyses of drugs and metabolites in biofluids. To date, there has been no quantitative LC/MS–MS methodology published for the analysis of ODN. Herein, we describe the development, validation and application of a sensitive, selective and robust LC/MS–MS assay for the quantitative determination of ODN in human plasma.

2. Materials and methods

2.1. Chemicals and reagents

Reference standards for ODN (purity >99%) and stable isotope $^{13}C_6$-labeled ODN internal standard (purity >97%) were synthesized at Merck Research Laboratories. The structure of ODN is shown in Fig. 1A. The stable isotope labeled internal standard (IS) [$^{13}C_6$]ODN was synthesized with C-13 distributed over the six carbons within the phenyl ring bearing the methylsulfonyl functional group (Fig. 1B). Control human plasma with K_2EDTA as the anti-coagulant was purchased from Biological Specialty Corporation (Colmar, Pennsylvania, USA). Formic acid, ammonium hydroxide concentrated solution (28–30%, w/w), ammonium formate, acetonitrile, and methyl t-butyl ether were obtained from Fisher Scientific (Waltham, Massachusetts, USA). All reagents and solvents were of analytical or HPLC grade.

2.2. Instrumentation

For the LC/MS–MS analysis, Shimadzu (Kyoto, Japan) LC-10ADVp pumps connected to a SIL-HTc autosampler were coupled with an Applied Biosystem/MDS Sciex (Ontario, Canada) API 4000 tandem mass spectrometer equipped with a turbo-ion spray source. A Packard MultiPROBE robotic liquid handler (PerkinElmer, Waltham, Massachusetts, USA) was used for plasma sample transfer. A Quadra 96 robotic work station (Tomtec, Hamden, CT, USA) was used for the partially automated liquid–liquid extraction.

2.3. Solution preparation

Stock solutions were prepared at a concentration of 1 mg/mL in 90/10 (v/v) acetonitrile/water containing 0.1% (v%) formic acid. Working solutions were diluted from stock solutions with 50/50 (v/v) acetonitrile/water to concentrations of 5000, 1000, 500, 100, 50, 25, 10 and 5 ng/mL. Solutions used in the preparations of quality control (QC) samples were prepared by dilutions of the stock solutions to concentrations of 30,000, 3000 and 120 ng/mL. A working solution of the internal standard (IS) was prepared at a concentration of 100 ng/mL. All solutions were stored at −20 °C and brought to room temperature before each use. Stability of ODN stock and working solutions were evaluated by comparing analyte-to-IS peak

area ratios of the stability assessment solutions with those of freshly prepared solutions. ODN was found to be stable in solution (difference in area ratio ≤ 7%) for at least 6 h at ambient temperature, and for up to 26 months when stored at −20 °C.

Plasma standards were prepared by spiking the appropriate volume of working solutions into control human plasma. The plasma standards were prepared on the day of sample analysis, at concentrations of 0.5, 1, 2.5, 5, 10, 50, 100 and 500 ng/mL. In each analytical run, control blank samples with and without spiking the IS working solution were assayed to ensure lack of interferences from the control matrix and the IS. Plasma QCs were prepared at concentrations of 1.2, 30 and 300 ng/mL by dilution of QC working solutions with control plasma. For evaluating ODN stability in plasma at different storage temperatures, one set of QCs was stored at −20 °C and another set at −70 °C.

2.4. Liquid–liquid extraction

Plasma samples were thawed and mixed thoroughly prior to analysis. Three hundred microliter aliquots of plasma were transferred to the wells of a clean 96-well plate (Arctic White LLC, Bethlehem, Pennsylvania, USA) using a Packard MultiPROBE liquid handler, followed by addition of 30 μL IS working solution to each of the samples. For control blank samples, an aliquot (30 μL) of 50/50 acetonitrile/water was added instead of IS. An aliquot of 150 μL of 0.15 M ammonium hydroxide was added to each sample, after which the samples were gently mixed. Methyl t-butyl ether (0.9 mL) was added as the liquid extraction solvent. The assay plate was sealed with a 96-well plate mat (Arctic White LLC, Bethlehem, Pennsylvania, USA) and rotated for 10 min to achieve thorough mixing. Then, the assay plate was centrifuged at 1500 × *g* for 5 min. After phase separation, the organic layer was transferred to a clean 96-well injection plate, utilizing a Tomtec Quadra 96 liquid handling workstation. The organic solvent was subsequently evaporated under a gentle stream of nitrogen at approximately 37 °C. Finally, the extracted samples were reconstituted in 500 μL of a solution containing 50/50 (v/v) acetonitrile/1 mM ammonium formate (pH 3). Depending upon instrument sensitivity (signal-to-noise ratio ≥ 5 at LLOQ concentration was required), a volume of 5 or 10 μL of reconstituted sample extract was injected into the LC/MS–MS system for analysis.

2.5. Liquid chromatography conditions

A Phenomenex Luna C18 (50 mm × 2.0 mm, 5 μm) (Phenomenex, Torrance, California, USA) analytical column was utilized. Mobile phase components A and B were 0.1% formic acid (in water) and acetonitrile, respectively. The analyte and its IS were separated using isocratic elution conditions with 65%B, a flow rate of 0.2 mL/min, and ambient column temperature. After analyte elution, the column was flushed with acetonitrile for 1 min, at a flow rate of 0.75 mL/min, and then re-equilibrated with 65%B for 1 min at 0.75 mL/min before the subsequent sample injection. A diverter valve was utilized to direct the flow from the LC column to the MS ion source only during the time period when the analyte and IS eluted, and to the waste for the rest of the run time. To maintain a stable spray, a makeup flow containing 65/35 (v/v) acetonitrile/0.1% formic acid was introduced into the ion source at a flow rate of 0.2 mL/min during the period when the column flow was directed to waste. The overall run time, including analyte elution, column flushing step and re-equilibration time, was approximately 4.5 min. The autosampler temperature was set at 5 °C.

2.6. Mass spectrometric method

A PE Sciex triple quadrupole mass spectrometer, API 4000, with turbo-ion spray source was interfaced with the LC system. It was operated in positive ionization and selected reaction monitoring (SRM) mode for monitoring selected precursor and product ions for ODN and IS. Mass spectrometric parameters were optimized as follows: The turbo spray temperature was 500 °C; the ion spray voltage was 4500 V, the curtain gas (N_2) flow was 30 psi; gas 1 (nebulizer gas, N_2) and gas 2 (auxiliary gas, N_2) were at 30 and 50 psi, respectively; collision-activated dissociation (CAD) gas (N_2) was set at 6 psi; the declustering potential (DP) was 58.56 V; the entrance potential (EP) was 10 V; the collision energy (CE) was 41.10 V; and the collision cell exit potential (CXP) was 18.17 V. Ion pairs *m*/*z* 526 → 313 and 532 → 319 were monitored for the analyte and IS respectively, with dwell time of 300 ms for each ion pair. The mass analyzers Q1 and Q3 were operated at unit mass resolution.

2.7. Data analysis

Mass spectrometric data were acquired and processed using software Analyst version 1.4.2 (Applied Biosystem/MDS-Sciex, Canada). Calibration curves were constructed by plotting the peak area ratio of analyte to internal standard versus analyte nominal concentration. Analyte concentrations were calculated using a $1/x$ weighted linear least-square regression analysis of the standard curve. Accuracy (%) was expressed by [(mean observed concentration)/(nominal concentration)] × 100. Precision (%) was calculated by [(standard deviation)/(mean observed concentration)] × 100.

3. Results and discussion

To support clinical pharmacokinetic studies, an LC/MS–MS assay was developed and validated for quantitative analysis of ODN in human plasma. Our objective was to develop a reliable and robust assay with a broad dynamic range (0.500–500 ng/mL) and relatively high throughput that would be suitable for long-term routine clinical pharmacokinetic sample analysis.

3.1. Chromatography

ODN and its IS were retained on the column (retention factor, k', approximately 1.5), with good separation from interfering endogenous compounds. The LC column was flushed with acetonitrile after each sample injection, to remove residual hydrophobic interferences. A diverter valve was applied to minimize potential contamination of the MS ion source. The variations in column backpressure and MS sensitivity were minimal even after hundreds of sample injections, which allowed the analysis of large clinical sample batches.

3.2. Mass spectrometry

Mass spectrometric fragmentation was determined by infusing the neat analyte working solution directly into the turbospray ion source. The protonated molecular ion $[M+H]^+$ (*m*/*z* 526) was observed via MS scan in the first quadrupole filter (Q1). When the parent ion was subjected to collision induced fragmentation, *m*/*z* 313 was found to be the predominant product ion, as shown in Fig. 1A. Similarly for the $^{13}C_6$-labeled IS, it was observed that ion pair *m*/*z* 532 → 319 was predominant under selected reaction monitoring (SRM) mode, and Fig. 1B shows the product ion mass spectrum for the IS.

Table 1
Extraction recovery assessment for ODN and $^{13}C_6$-ODN (IS).

Analyte	Standard conc. in human plasma (ng/mL)	Mean extraction recovery (%, $n = 6$)	CV (%)
ODN	1.00	102	11.7
	50.0	93.7	5.68
	500	101	4.95
$^{13}C_6$-ODN	10.0	98.0[a]	7.63

[a] $n = 18$.

3.3. Extraction recovery

Sample preparation was based on a semi-automated liquid–liquid extraction (LLE) procedure. LLE is a traditional sample preparation methodology suitable for hydrophobic molecule bioanalysis. In this assay, the LLE procedure was conducted in a 96-well plate format to make the liquid transfer steps amenable to automation. Methyl t-butyl ether (MTBE) was chosen as the organic solvent for extraction.

To evaluate the extraction recovery, the analyte peak areas for reconstituted extracts of plasma samples pre-spiked with analyte at 1, 50 and 500 ng/mL and IS at 10 ng/mL were compared with those samples spiked with analyte and IS at the same concentrations post-extraction. The extraction recovery was found to be high and reproducible under basic conditions, with mean recovery of approximately 99% for ODN, and 98% for the IS (Table 1).

3.4. Sensitivity

Based on the pharmacokinetic characteristics of ODN and the clinical doses being studied (ranging from 0.5 to 600 mg), a lower limit of quantitation (LLOQ) of 0.500 ng/mL was required. To assess assay performance at this concentration, five replicates of samples at 0.500 ng/mL were assayed. The mean observed concentration was 0.470 ng/mL, with a precision of 4.52% and an accuracy of 94.1%. These results demonstrate acceptable assay performance at the LLOQ.

3.5. Selectivity

Assay selectivity was examined by analyzing control blank plasma samples from six different sources. In the ion chromatograms, no interfering peaks were present at the retention times of ODN and the IS in the MS/MS ion channels being monitored. Lack of interference was further confirmed in the pre-dose samples collected from clinical studies. Representative chromatograms are presented in Fig. 2.

Lack of "crosstalk" between the analyte and IS detection channels was demonstrated. When plasma samples containing the highest concentration (500 ng/mL) of the standard curve in the absence of IS were assayed, no peak was observed in the IS detection channel. Similarly, in the chromatograms for plasma samples containing IS at the working concentration (10 ng/mL), there were no peaks observable from the analyte channel.

Recent regulatory guidelines have recommended assessing the impact of concomitant medications on bioanalytical assay performance [8]. In an effort to demonstrate assay specificity in the presence of commonly used medications that could potentially be co-administered with ODN, ODN plasma samples (1.20 ng/mL, $n = 5$) were spiked with 11 medications at concentrations approximately equal to or greater than the literature reported maximum plasma concentration (C_{max}) (Table 2), and then subjected to analyses along with control samples prepared at the same analyte concentration in absence of the 11 co-meds. Analyte-to-IS peak area ratios of co-med spiked samples were compared with those of control. The difference in peak area ratios between the two sets of samples was −0.04%.

Table 2
Concentrations of concomitant medications for the assay specificity test.

Drug name	Spiking concentration
Acetaminophen (Tylenol)	30 μg/mL
Acetylsalicylic acid (Aspirin)	30 μg/mL
Caffeine	10 μg/mL
Dextromethorphan (DXM)	3 ng/mL
Ibuprofen	80 μg/mL
Nicotine	30 ng/mL
Pheniramine	1 μg/mL
Phenylephrine	60 ng/mL
Pseudoephedrine	350 ng/mL
Diphenhydramine (Benadryl)	100 ng/mL
Ketoconazole	25 μg/mL

3.6. Matrix effect

The composition of the sample matrix could vary significantly from subject to subject and from time point to time point. Studies have shown that the variability in matrix content could potentially undermine the performance of LC/MS–MS assays [9–13]. In this respect, matrix effect for ODN was evaluated to ensure assay ruggedness.

Absolute matrix effect was assessed for ODN at concentrations of 1.00, 50.0 and 500 ng/mL ($n = 6$ at each concentration level). The analyte peak areas in the extracted samples were compared with those obtained in the neat solution. The mean calculated matrix effect was 108% ($n = 3$, CV = 1.45%) for ODN, and 104% ($n = 18$, CV = 1.55%) for IS, suggesting no significant ion suppression or enhancement.

Given that the analyte concentrations are typically derived from a standard curve prepared from a single source of matrix, variability in slope values regressed from standard curves prepared in six different sources of plasma was evaluated. The mean slope was found to be 0.113 with precision of 3.01% ($n = 6$), reassuring that minimal assay liabilities from matrix effect was expected [11].

3.7. Intra-run and inter-run variability

Intra-run assay variability was assessed by analyzing six sets of standard curve samples (0.500–500 ng/mL), each prepared in a different lot of plasma, and four replicates of low, medium, and high quality control samples (QCs) prepared at ODN concentrations of 1.20, 30.0, and 300 ng/mL, respectively. The precision and accuracy data for the back-calculated standards are shown in Table 3, and QC data are shown in Table 4.

The data for six sets of standard curve samples (Table 3) was utilized to assess the overall effect of assay and sample matrix variability on assay performance. The accuracy was between 90.9 and

Table 3
Accuracy and precision for back-calculated concentrations of six sets of standard curve samples, each prepared in a different lot of human plasma.

Nominal conc. (ng/mL)	Mean observed conc.[a] (ng/mL)	Accuracy (%)	Precision (%)
0.500	0.455	90.9	4.97
1.00	0.969	96.9	3.80
2.50	2.46	98.4	2.71
5.00	5.05	101	2.46
10.0	10.6	106	2.48
50.0	51.0	102	2.54
100	106	106	2.41
500	492	98.4	3.44

[a] $n = 6$.

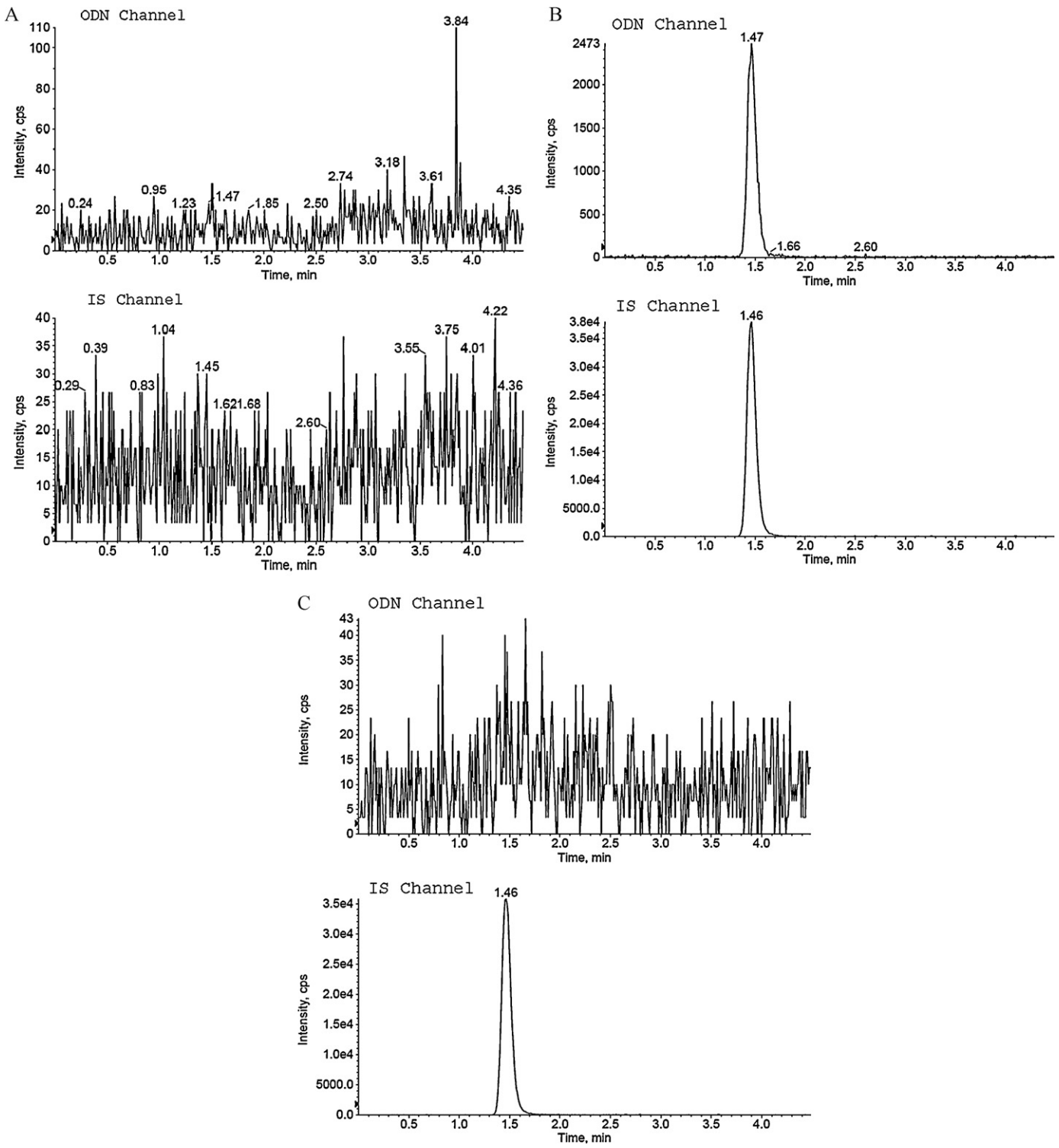

Fig. 2. Representative chromatograms: (A) extracted blank plasma; (B) extracted plasma sample prepared at lower limit of quantitation concentration (0.5 ng/mL) spiked with 10 ng/mL of internal standard; (C) extracted pre-dose sample spiked with 10 ng/mL of internal standard.

106% of the nominal concentrations, and precision between 2.41 and 4.97%. A representative standard curve is depicted in Fig. 3. The regression coefficient, *r*, was found to be 0.9996.

For QC samples, the intra-run variability was within 3.54%, and the accuracy for mean observed QC concentrations was between 95.6 and 102% of their nominal values (Table 4). Inter-run variability was assessed by examining the individual QC concentrations from 12 different analytical runs ($n = 2$ at each concentration per run) in study sample analysis. The data in Table 4 show that inter-run precision was between 3.65 and 5.88%, and accuracy was between 99.2 and 106%.

3.8. Dilution integrity

It is probable that some plasma PK samples may contain analyte at concentrations exceeding the upper limit of quantitation (ULOQ)

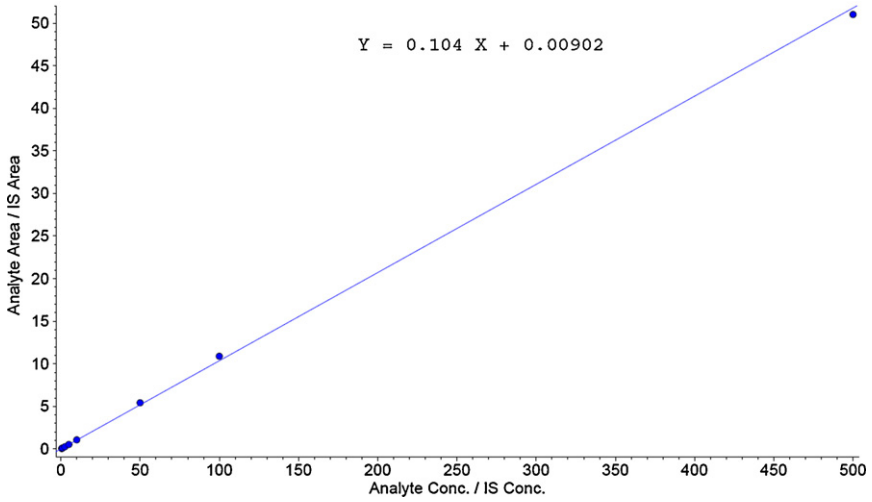

Fig. 3. Representative standard curve.

of the assay; therefore, assay performance following sample dilution was evaluated. Dilution assessment samples were prepared at the concentration of 3000 ng/mL, 6-fold higher than the assay ULOQ (500 ng/mL). To imitate the actual sample handling conditions, the samples were stored at −70 °C for at least 24 h prior to testing. On the day of analysis, these samples were thawed and diluted with control human plasma by 10-fold, to bring the analyte concentration into the calibration range. The mean observed concentration of diluted samples was 95.7% of the nominal concentration, with a precision of 1.6% ($n = 3$).

3.9. Long-term stability in human plasma

During a clinical trial, human plasma samples are typically collected and frozen at the clinical sites, then shipped to the bioanalytical laboratory for analysis at a frequency per assay and study needs. To demonstrate sample integrity after sample collection and during storage prior to sample analysis, a batch of plasma QC samples were prepared, frozen at the intended storage temperature (−20 or −70 °C), and assayed periodically to check analyte concentrations against their nominal and initial analysis concentrations. The long-term plasma storage stability at −20 and −70 °C was tested for up to 38 months and no significant analyte degradation was observed (Table 5).

Table 4
Quality control (QC) accuracy and precision.

Intra-run QC accuracy and precision			
Nominal conc. (ng/mL)	Mean observed conc.[a] (ng/mL)	Accuracy (%)	Precision (%)
1.20	1.23	102	3.54
30.0	28.7	95.6	3.44
300	294	97.9	2.04
Inter-run QC accuracy and precision			
Nominal conc. (ng/mL)	Mean observed conc.[b] (ng/mL)	Accuracy (%)	Precision (%)
1.20	1.19	99.2	5.88
30.0	31.8	106	3.68
300	314	105	3.65

[a] $n = 4$.
[b] $n = 24$.

3.10. Short-term stability

Short-term stability tests were performed to demonstrate the sample integrity under various sample preparation, analysis and short-term storage conditions, as described below.

3.10.1. Freeze–thaw stability

Analyte freeze–thaw stability (from −20 or −70 °C to room temperature) was evaluated. Plasma samples at ODN concentrations of 1.20, 30.0 and 300 ng/mL were subjected to three freeze–thaw (FT) cycles, with each cycle consisting of exposure to the intended storage temperature for at least 24 h followed by an unassisted thaw to reach room temperature. The stability assessment samples were assayed along with control, plasma samples exposed to one FT cycle. The differences from control were between −1.3 and 2.3% for samples undergoing three FT cycles from −20 °C to room temperature, and between −0.9 and 2.8% for samples with three FT cycles from −70 °C to room temperature (Table 6). It is therefore considered that there was no significant change in analyte plasma concentrations after up to three FT cycles.

3.10.2. Bench-top stability

To test analyte stability for the duration of time expected for the extraction procedure, stability QC samples prepared at 1.20, 30.0 and 300 ng/mL were placed at ambient temperature and exposed to normal lab white light for a period of 4 h. A separate set of QCs was thawed after the stability QCs were kept at ambient temperature for the intended length of time. Both sets of QCs were assayed within the same analytical run and the mean observed concentrations were compared. Results demonstrated analyte stability under the testing conditions for at least 4 h (Table 6).

3.10.3. Processed sample stability

Processed sample stability was evaluated to ensure analyte stability for the period of anticipated run time as well as for delayed injections in unanticipated situations, such as instrument failure. Extracted plasma QC samples were kept in the autosampler for a period of 80 h, and concentrations were determined based on the regression of a freshly prepared standard curve. No analyte degradation was observed for the test period (Table 6).

3.10.4. Reinjection reproducibility

The reproducibility of re-injecting the extracted samples was evaluated. Precision and accuracy results from both the initial injection and re-injection of the same samples from the entire analytical

Table 5
Analyte stability in human plasma after storage for up to 38 months at −20 °C. Numbers in parentheses are accuracy (%) and coefficients of variation (CV%), respectively.

Storage temperature	Nominal conc. (ng/mL)	Mean observed conc.[a] for control (Day 1) (ng/mL)	Mean observed conc.[a] for 16-month stability samples (ng/mL)	Mean observed conc.[b] for 38-month stability samples (ng/mL)
−20 °C	1.20	1.14 (94.8, 5.9)	1.13 (93.8, 0.9)	1.20 (100, 2.7)
	30.0	27.0 (90.0, 3.0)	30.3 (101, 2.1)	30.6 (102, 1.8)
	300	286 (95.4, 2.5)	301 (101, 1.1)	306 (102, 1.3)
−70 °C	1.20	1.23 (102, 3.6)	1.12 (93.3, 3.2)	1.34 (111, 24.0)[c]
	30.0	28.7 (95.6, 3.4)	29.6 (98.7, 1.4)	29.6 (98.8, 1.1)
	300	294 (97.9, 2.0)	301 (100, 1.9)	300 (100, 1.5)

[a] $n = 4$.
[b] $n = 5$.
[c] An observed value of 1.89 ng/mL for one of the five replicates at the nominal concentration of 1.20 ng/mL has been included in the statistical calculations. According to Dixon's Q-test results, this value can be rejected as an outlier at 90% confidence interval. The observed Q value was 0.709, greater than the critical Q value of 0.642 ($\alpha = 0.10$, $N = 5$). If this outlier is excluded, the statistics are: mean conc. 1.20 ng/mL; accuracy 99.8%; CV 8.0%.

Table 6
Difference from control (%) during analyte stability and re-injection reproducibility assessment.

Nominal conc. (ng/mL)	Three freeze–thaw cycle stability	Four hours ambient temperature stability	Eighty hours processed sample stability	Re-injection reproducibility
1.20	2.2[a], −0.9[b]	0.0	0.8	−0.8
30.0	−1.3[a], 2.8[b]	3.0	−1.0	1.6
300	2.3[a], −2.2[b]	−1.9	−0.04	2.7

[a] Frozen temperature = −20 °C.
[b] Frozen temperature = −70 °C.

run were compared and found to be consistent. The %differences between the initial run and re-injections are shown in Table 6.

3.11. Study sample analysis

This assay has been cross-validated by multiple analysts on multiple instruments in multiple laboratories, and applied to clinical bioanalysis for studies from phase I to III of drug development. In one of the clinical pharmacokinetic studies, ODN plasma concentrations in healthy human subjects administered with a single, oral, 25 mg dose of ODN after an overnight fast (at least 8 h) were monitored for 14 days post-dose. Representative chromatograms from study samples collected at 9 and 336 h post dose are shown in Fig. 4. The concentration–time profiles for 12 individual subjects are depicted in Fig. 5. The mean observed C_{max} was 87.9 ng/mL, and C_{max} occurred at a median T_{max} of 9 h post dose.

3.12. Incurred sample reproducibility (ISR)

Standards or QC samples are typically prepared by spiking the analyte(s) of interest *in vitro*. Thus, these samples may not contain certain elements present in the post-dose study samples such as prodrug, metabolites, dosing vehicles and/or other matrix interferences [14]. As a result, the impact of these additional components on assay reproducibility may not be revealed by simply testing standards and QCs in the validation studies. To address this potential liability, confirmatory reanalysis of incurred samples has been recommended, as a means to evaluate the assay performance to a greater extent [15].

A subset of post-dose clinical ODN plasma samples (thirty-two samples in total), collected from the complete pharmacokinetic profiles of two individual patients, were selected and re-analyzed in an independent analytical run. Each of the selected subjects was from a special population study, which were renal and hepatic insufficiency study, respectively. Presumably, these subjects may

Table 7
Incurred sample reproducibility.

Subject ID	Time point (h)	Original conc. (ng/mL)	Reanalysis conc. (ng/mL)	Difference[a] (%)
1	1	20.7	20.2	−2.6
1	2	43.9	41.5	−5.5
1	4	92.5	89.7	−3.1
1	6	153	143	−6.5
1	9	150	139	−7.6
1	12	142	129	−9.5
1	16	135	134	−0.8
1	24	136	127	−7.1
1	32	150	135	−9.9
1	48	146	145	−0.8
1	72	143	132	−7.5
1	96	116	110	−5.9
1	120	85.9	83.8	−2.5
1	168	56.8	52.5	−7.9
1	240	27.7	25.3	−9.2
1	336	16.3	15.5	−5.0
2	1	41.4	39.0	−6.1
2	2	98.8	91.3	−7.9
2	4	147	141	−4.0
2	6	142	140	−1.2
2	9	143	132	−8.4
2	12	131	122	−6.7
2	16	119	112	−5.5
2	24	121	119	−1.6
2	32	94.8	86.7	−9.0
2	48	95.9	93.2	−2.8
2	72	76.2	70.4	−7.9
2	96	62.4	59.2	−5.2
2	120	49.1	45.0	−8.8
2	168	34.1	32.4	−5.1
2	240	21.5	19.5	−9.9
2	336	15.2	14.8	−2.7

[a] Difference% = (reanalysis conc. − original conc.) × 100/average conc.; average conc. = (original conc. + reanalysis conc.)/2.

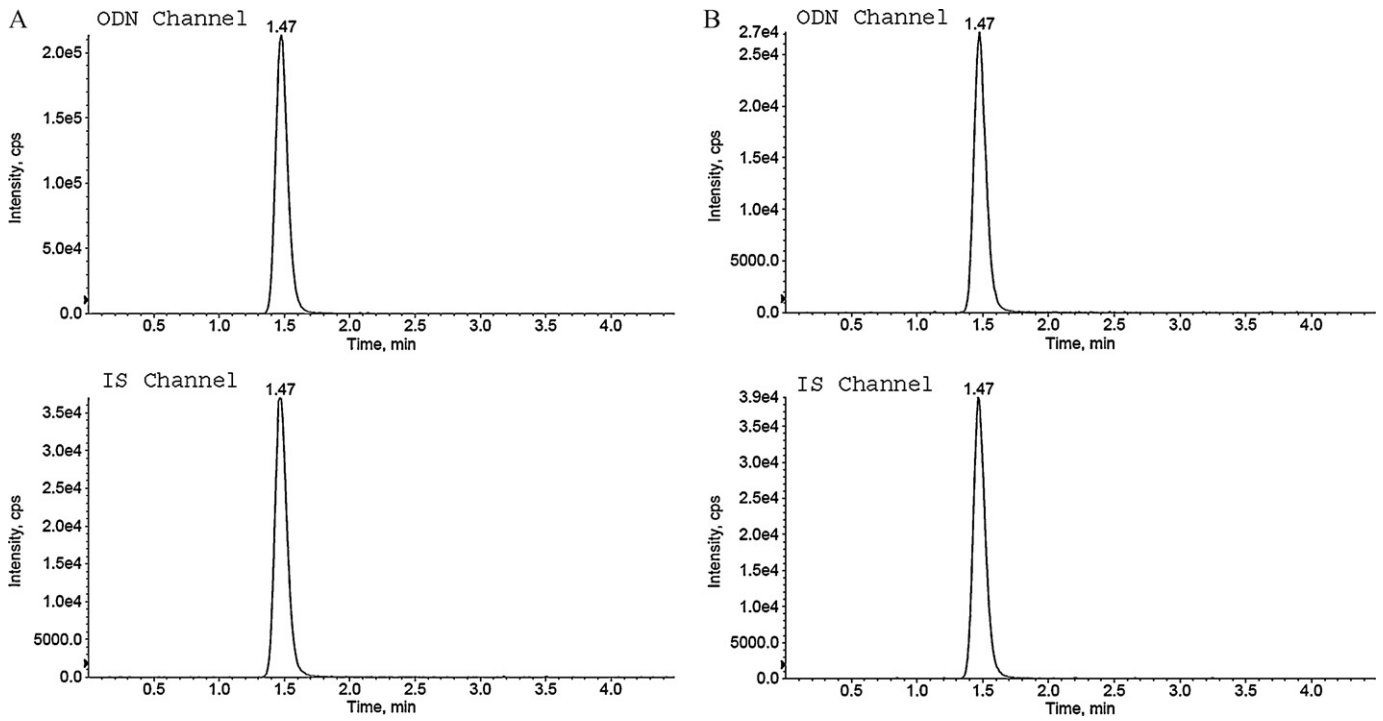

Fig. 4. Representative chromatograms for extracted clinical study samples spiked with 10 ng/mL of internal standard. The clinical samples were collected at 9 and 336 h post a 25-mg, single oral dose of ODN. (A) 9-h sample; (B) 336-h sample.

exhibit pronounced differences in ODN metabolic and pharmacokinetic characteristics. The selected samples contained analyte at concentrations spreading roughly over the entire anticipated concentration range for the studies, and the time points of the selected samples covered the entire post-dose sample collection time frame.

The re-analysis results were compared with the initial analyses results, and have shown acceptable data consistency, with 100% of the reanalysis data deviating less than 10% (ranging from −0.8 to −9.9%) from their original values. Upon inspection of the percentage of difference data (Table 7), there seems to be a very slight negative bias in the reanalysis results. This bias is likely attributed to inter-run assay variability, and possibly the limited number of subjects being selected for ISR evaluation. Overall, the concentration data appear to be reproducible.

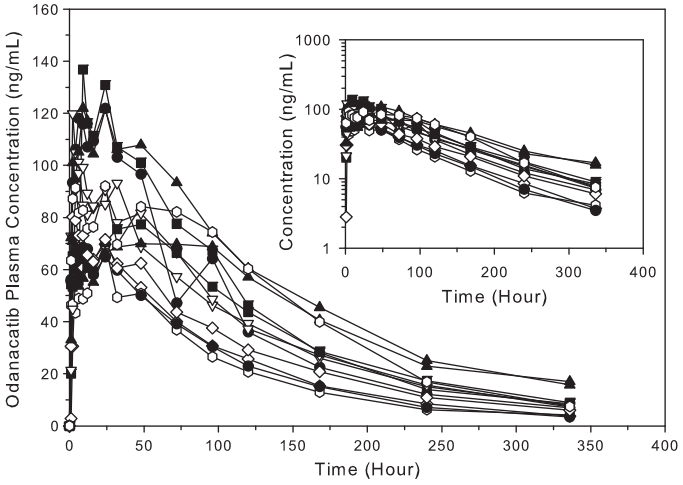

Fig. 5. Representative ODN plasma concentration–time profiles (linear and log scale) for healthy human subjects (12 in total) administered with a single 25-mg oral dose of ODN.

4. Conclusions

This manuscript describes an LC/MS–MS assay following a semi-automated liquid–liquid extraction for quantitative analysis of odanacatib in human plasma. The validated assay calibration range was from 0.500 to 500 ng/mL. Assay specificity and reproducibility were rigorously assessed and validated in accordance with regulatory guidelines for clinical bioanalytical methods. This methodology has been utilized for routine sample analysis in numerous clinical studies, and has proved to be accurate, robust, and fairly cost-effective. It is reasonable to draw the conclusion that this assay is well suited for ODN plasma sample analysis in support of clinical trials at various stages of drug development.

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