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A sensitive LC–MS/MS method for quantification of a nucleoside analog in plasma: Application to *in vivo* rat pharmacokinetic studies

Olivier Heudi*, Samuel Barteau, Franck Picard, Olivier Kretz

Novartis Pharma AG, DMPK/Bioanalytics, Forum 1 Novartis Campus, CH-4056 Basel, Switzerland

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ABSTRACT

A LC–MS/MS method was developed and validated for determination of nucleoside analog (NA) in rat plasma. The method run time was 6 min and the limit of quantification (LOQ) was estimated at 100 pg/mL. The extraction procedure consisted on plasma samples protein precipitation with an acetonitrile solution which contained the stable isotope labeled internal standard (IS). Chromatography was performed on a newly developed C_{16} column (150 mm × 4.6 mm, 5 μm) in order to avoid the use ion pair salts. The samples were eluted at 0.8 mL/min with a gradient of mobile phase made of water and acetonitrile both acidified with 0.5% acetic acid and 0.025% trifluoroacetic acid (TFA). A tandem mass spectrometer was used as a detector for quantitative analysis. Intra-run and inter-run precision and accuracy within ±15% were achieved during a 3-run validation for quality control samples at four concentration levels in rat plasma, over a concentration ranging between 0.1 and 1000 ng/mL. The data indicate that our LC–MS/MS assay is an effective method for the pharmacokinetics study of NA in rat plasma.

1. Introduction

Several virus infections including hepatitis, human immunodeficiency and herpes simplex, are currently treated with nucleoside analog (NA) [1–7]. Recently, it has been shown that NA can decrease viremia in a dose-dependent manner in mice infected with dengue virus [8]. In this respect, a new program based on the selection of NA with an anti-dengue virus activity was initiated. In order to support such a program, the development of rapid and sensitive analytical method for NA quantification in biological fluid is needed.

LC–MS/MS is a well-established method in bio-analysis due to its inherent specificity, sensitivity and speed. Reversed-phase columns are commonly used for the compounds separation in these developed LC–MS/MS methods. However, NA is not retained on such columns because of its polar nature. Anion exchange columns which yield excellent peak shape and predictable retention times for nucleotides and NA have been also tested [7], but the low volatility of the salt used for the elution of compounds is not always compatible with MS. Ion-pairing salts with more volatility such as *N*,*N*-dimethylhexylamine (DMHA) were successfully employed for the analysis of NAs by MS [9,10]. However, the use of DMHA increases the analysis run time due to the column equilibration and this generates LC–MS/MS methods that are not suitable for achieving fast bio-analysis. New types of LC-columns that can tolerate high amount of aqueous in the mobile phase (up to 100% aqueous phase) can offer an alternative for the separation of NA [11]. However, aqueous mobile phase has been speculated to yield poor ionization efficiency when using MS detection. Thus, in the later work, a post-column addition of acetonitrile was used to increase the ionization efficiency in the MS source. This makes the method complex as an additional pump and parameters optimization are required.

Extraction methods for NA in biological matrices such as blood or plasma remain challenging. Solid phase extraction (SPE) is not commonly used because of the low recovery of NAs obtained with this approach [11]. Protein precipitation with or without liquid/liquid extraction has been mostly applied. However, with a limit of quantification ranging from 0.5 to 10 ng/mL the proposed methods [11,12] were not suitable to analyze samples with low concentrations of NA. The purpose of the present work was to develop and validate a rapid and sensitive LC–MS/MS method for the quantification of one of our promising anti-dengue NA drug candidate also named cpd-1 (Fig. 1) in plasma. Hence, a LC–MS/MS method with a limit of quantification of 0.1 ng/mL and a run time of 6 min was developed and validated in rat plasma by applying a simple extraction procedure and by using new type of column in order to shorten the analysis time. This method was then used to support *in vivo* rat pharmacokinetic studies of cpd-1 after an intravenous or oral dose of [^{14}C]cpd-1.

* Corresponding author. Tel.: +41 79 53 59 611; fax: +41 61 696 85 84.
E-mail address: Olivier.heudi@novartis.com (O. Heudi).

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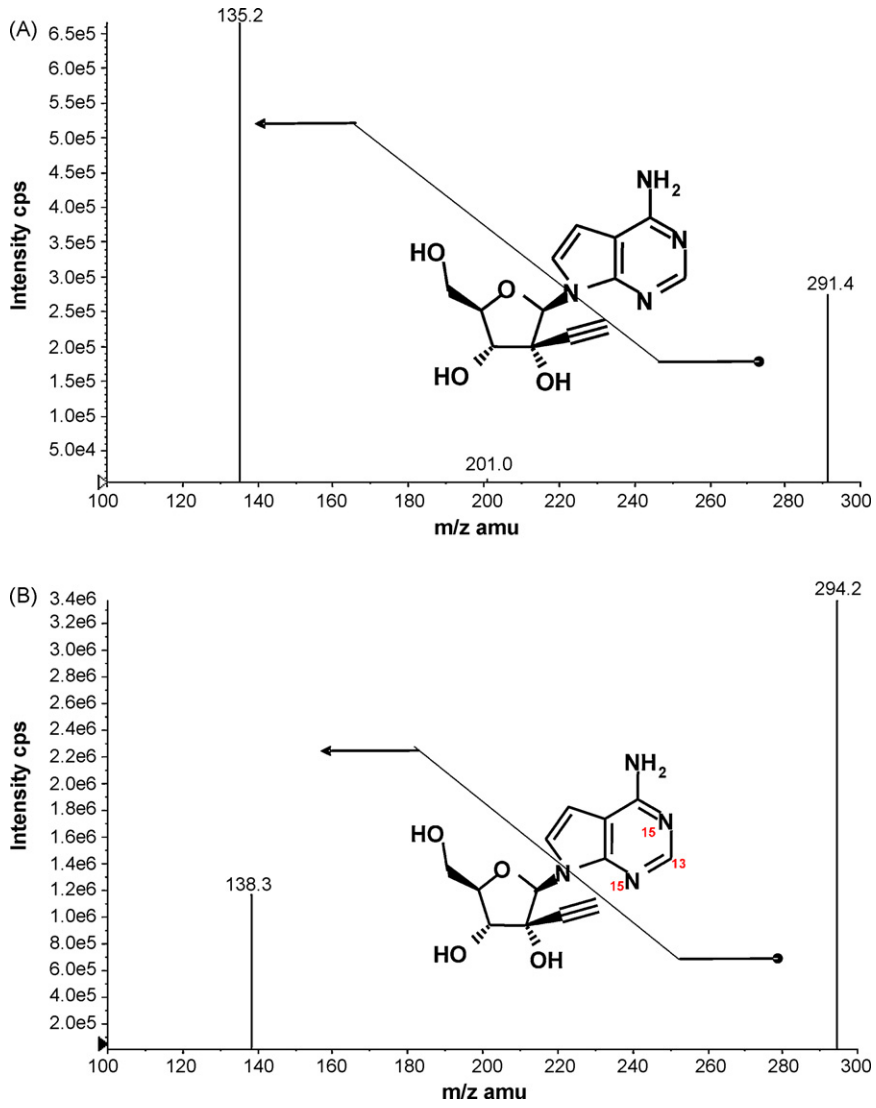

Fig. 1. Representative ion mass spectra and proposed fragmentation for cpd-1 (A) and that of its internal standard (IS) (B).

2. Experimental

2.1. Chemicals and reagents

Acetic acid (100% anhydrous), methanol, acetonitrile and tetrahydrofuran were obtained from Merck KGaA (Darmstadt, Germany). TFA was obtained from Fluka (Buchs, Switzerland). cpd-1, stable isotope labeled IS and [^{14}C]cpd-1 were synthesized in-house.

2.2. Preparation of stock and working solutions

Individual cpd-1 stock was prepared in water/acetonitrile (50:50, v/v) to give a final concentration of 182 μg/mL. Individual working calibration standard (Cs) solutions with concentrations of 10.0, 303, 10,100, 35,350, 65,650 and 101,000 ng/mL were prepared after serial dilutions of the stock solution with methanol. The working Quality Control sample (QCs) solutions with concentrations of 10.0, 30.0, 20,200 and 75,750 ng/mL were prepared in the same manner. The working solutions were freshly prepared on each day of the analysis.

2.3. Preparation of Cs and QCs

Two different batches of plasma were used for the preparation of Cs and QCs. The Cs samples were prepared by spiking 10 μL of each cpd-1 individual working Cs solution into 1000 μL of blank rat plasma. This yielded Cs concentrations of 0.10 (LOQ), 3.00, 100, 350, 650 and 1000 ng/mL. The QCs were prepared in the same manner to give final concentrations of 0.10, 0.30, 200 and 750 ng/mL.

2.4. Sample extraction

Plasma samples were extracted in a 96-Deepwell plate. To 100 μL rat plasma was added 200 μL acetonitrile containing the stable isotope labeled IS at a concentration of 69 ng/mL. For the blank plasma samples, 200 μL acetonitrile was added in the plate. The plate was then shaken for approximately 60 s before being centrifuged at approximately 2250 × *g* for 10 min at 4 °C. The plate was then placed on the auto-sampler and 20 μL was injected (injection loop 50 μL) for LC–MS/MS analysis.

2.5. Animal studies and sample handling for PK

Rat HanWistar (Charles River), three animals per time point were given either 5 mg/kg intravenously or 25 mg/kg orally of [^{14}C]cpd-1 formulated in 100 mM citrate buffer, pH 3.5. Following single dose administration, 200 μL blood was collected in heparinized micro-pipettes (Microcaps, Drummond Scientific Co.,

Broomall, PA) from each rat by tail bleeding at 0.083, 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48, 72, 96, and 168 h post-dose without removing the animals from the metabolism cages. The total blood volume collected did not exceed 1% of the animal body weight. The blood sample was spun to separate the plasma. A volume of 20 μL of each plasma sample was removed for radioactivity analysis. The remaining plasma volume (~75 μL) was stored in separate polypropylene tubes at −20 °C until analysis of cpd-1 by LC–MS/MS. Prior to the animal sample analysis, the plasma samples were diluted (1:1, v/v) with fresh rat plasma in order to have enough plasma volume for the sample extraction described in Section 2.4.

2.6. Analysis of radioactivity in plasma

An aliquot volume of 20 μL of each sample was transferred into individual 20 mL scintillation vials. An aliquot of 500 μL solvable was then added to each sample. The samples were incubated in a shaking water bath at 50 °C for 2 h. Thereafter, 15 mL of scintillation fluid was added to each vial and the samples were placed in the dark overnight to reduce chemiluminescence prior to counting.

2.7. LC–MS/MS conditions

Sample analysis was performed on a LC–MS/MS system consisting of an API 4000 triple quadrupole mass spectrometer equipped with a TurboIonSpray™ interface from Applied Biosystems (Foster City, CA, USA). The MS system was connected to a HTS CTC PAL auto-sampler (Zwingen, Switzerland) and to an Agilent 1100 pump system (Wilmington, DE, USA). Chromatographic separations were performed at a flow rate of 0.8 mL/min on a Dionex AcclaimPolar Advantage C_{16}, column 150 mm × 4.6 mm (Sunny Vale, CA, USA) equipped with a Phenomenex C_{18} Guard Cartridge 4.0 mm × 3.0 mm I.D. (Utrecht, The Netherlands). A binary gradient with a mobile phase consisting of water (A) and acetonitrile (B) was used for the LC-separation. The both mobile phases (A) and (B) were acidified with 0.5% acetic acid and 0.025% TFA. The elution gradient program was as follows: [time (min), (% mobile phase B): (0, 5) (1, 5) (3.5, 90) (4.5, 90) (5, 5) (6, 5)]. The column temperature was maintained at 30 °C using a column heater. The auto-sampler syringe and the injection valve were washed with 1.5 mL of tetrahydrofuran/methanol/water (1:1:1, v/v/v) containing 0.5% TFA and acetonitrile/water (4:1, v/v) to reduce carryover. The system was operated in electrospray positive ionization using SRM mode. The other MS conditions were as follows: turbo ion spray 4500 V; source temperature 700 °C; collision activated dissociation 5; curtain gas 30 psi; Gas1 40 psi; Gas2 40 psi; entrance potential 10 V; dwell time 150 ms; collision energy 31 eV; declustering potential 66 V (cpd-1) and 61 V (IS) and collision cell exit potential 8 V (cpd-1), and 10 V (IS).

3. Results and discussion

3.1. Method development and sample preparation

LC is widely used for the separation of NA in biological fluids. The LC methods have been improved for the retention of NA by using ion paring agent [9,10], underivatized silica [13,14] or new types of columns [11]. Thus, the column choice has a great influence on the separation of NA and on the success of a LC–MS/MS method. Consequently, a compromise between the hydrophilic and lipophilic characters of the column stationary phases has to be found. In the present study, four endcapped reversed-phase columns: YMC hydrosphere, Agilent Zorbax and Shiseido AQ and a new type of column: C_{16}Dionex Acclaim were compared on the basis of operating at low pH (pH < 3) as well as in the presence of high percentage of water in the mobile phase. The data of this comparison show that under our LC-conditions, the Dionex Acclaim and the Shiseido columns provide excellent results in terms of retention, peak shapes and column stability (data not shown). The Dionex Acclaim column was chosen for the present work. This column features a polar-enhanced stationary phase. This phase consists of a C_{16} functional group bonded to the surface of ultrapure silica using a sulfonamide group coupled to an ether linkage. Recent works [13,14] dealing with NAs LC-separation have been performed with mobile phase consisting of high organic, low aqueous phase and TFA. In these works the authors have shown an enhancement of the MS ionization efficiency of NA. Also, Xing et al. [15] demonstrated that TFA has a greater propensity to protonate nucleoside bases. In the present work TFA was added in the mobile phase used for the separation of cpd-1. In order to alleviate the sensitivity loss due to the use of TFA previously reported [11], acetic acid was added in mobile phase as suggested by a recent paper published by Shou and Weng [16]. Under our LC-conditions, TFA was used to control the mobile phase pH and to improve peak shapes of cpd-1 as illustrated by its sharp LC peak.

Protein precipitation followed by liquid/liquid extraction has been commonly applied for the extraction of NA in biological fluids [12]. Despite these multiple steps in the sample preparation, the LOQ obtained for the developed method is 0.5 ng/mL, which is, for some pre-clinical studies not enough to determine low concentration of NA. Different solvents have been used for the sample extraction and most of them are based on either acetonitrile or ethyl acetate. Recently, a mixture of methanol–acetonitrile has also been tested [11]. In the present study, several precipitating solutions were tested: a mixture of methanol–acetonitrile in which the percentage of acetonitrile varied was tested or a solution of acetonitrile containing acetic acid and/or TFA. With all tested solutions, we have always found lower NA extraction (data not shown). Under our LC-conditions we have observed a bad peak shape for cpd-1 when high percentage of methanol (>60%) was used in the precipitating solution. The best compromise between the recovery and the chromatography of cpd-1 was obtained when 100% acetonitrile was used in the precipitating solution.

3.2. Selectivity

Positive ion electrospray MS/MS product-ion spectra of cpd-1 and that of its labeled internal standard are shown in Fig. 1. For cpd-1, the most abundant product ion was observed at m/z 135 (Fig. 1A), which was due to the cleavage of the ribose moiety of the protonated molecule at m/z 291. Similar cleavage pattern was observed with the internal standard (Fig. 1B).

LC–MS/MS chromatograms of three lots of blank plasma showed no peak at the retention times of cpd-1 and IS, indicating that our method is highly selective. An example of SRM chromatograms of one blank extracted plasma is depicted in Fig. 2A (cpd-1 channel) and in Fig. 2B (IS channel). There was no interference between the IS and cpd-1 peak at the IS concentration used in the present work. Representative SRM chromatogram of blank plasma sample spiked with the IS (zero sample) at the concentration used in this study is depicted in Fig. 2C.

3.3. Sensitivity

The limit of quantification (LOQ) of the method was 100 pg/mL for cpd-1 when using a rat plasma extract volume of 0.1 mL. Representative SRM chromatogram of blank plasma sample spiked with cpd-1 at a concentration of 0.1 ng/mL (LOQ) is depicted in Fig. 3. As

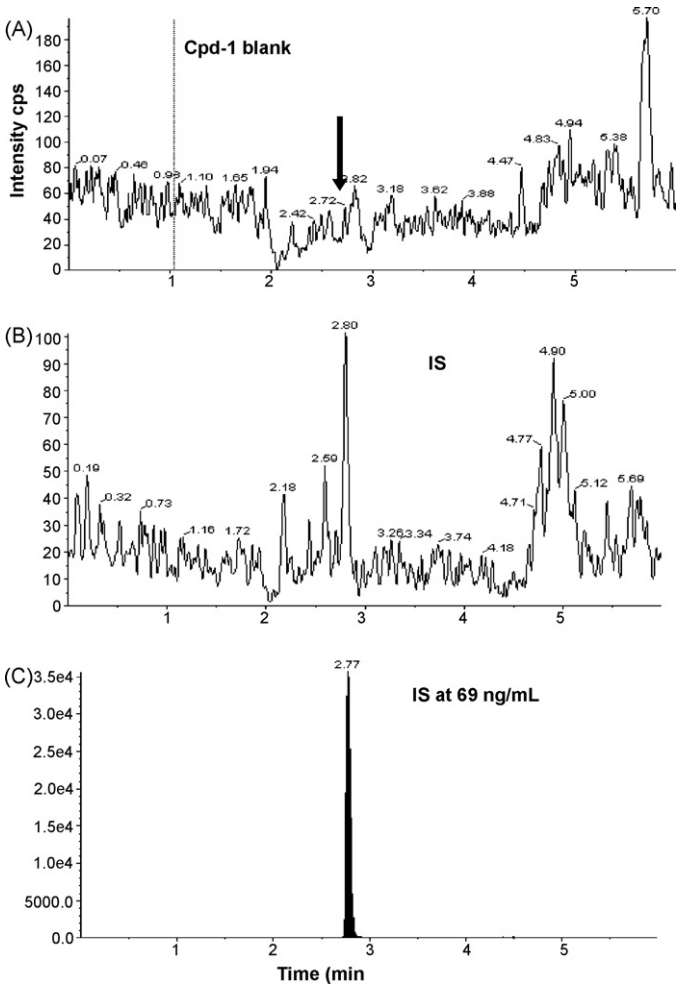

Fig. 2. Representative SRM chromatograms of extracted blank plasma sample without cpd-1 (m/z 291 → 135) (A) and $^{13}C^{15}N_2$-cpd-1, the IS (m/z 294 → 138) (B) or extracted blank plasma sample spiked with the IS (zero control sample) at a concentration of 69 ng/mL (m/z 294 → 138) (C). The arrow indicates the retention time of cpd-1.

can be seen, the signal of cpd-1 at the LOQ was well above the noise level.

3.4. Calibration

Plasma calibration curve was constructed using peak area ratios of cpd-1 to that of its IS and applying a weighted ($1/x^2$) least-squares quadratic regression analysis. Daily variation of calibration parameters obtained in rat plasma is shown in Table 1. As can be noticed, the regression coefficient (R^2) was above 0.995. The calibration curve parameters obtained on each of the 3 days were suitable for the quantification of cpd-1 in the samples during the intra- and inter-day validations and stability tests.

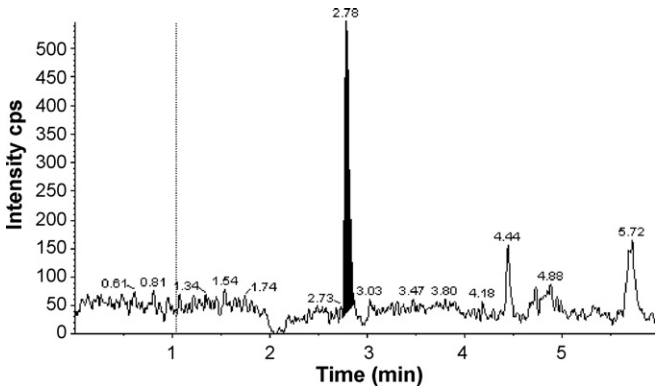

Fig. 3. Representative SRM chromatogram of extracted plasma sample spiked with cpd-1 at a concentration of 0.1 ng/mL (LLOQ) (m/z 291 → 135).

3.5. Precision and accuracy

Precision (expressed as percent relative standard deviation, %CV) and accuracy (expressed as percent error, %bias) were calculated for the four QCs (concentrations of 0.10, 0.30, 200 and 750 ng/mL). At least five replicates of each QC point were analyzed every day to determine the intra-day accuracy and precision. This process was repeated over 3 days in order to determine the inter-day accuracy and precision. The intra-run QCs accuracies were within the range ±20% at the LOQ and ±15% at the other concentration levels with at least 3/4 of the individual back-calculated values fulfilling the acceptance criteria (Table 2). The inter-run precision and accuracy data for QCs ranged between 2.3 and 12.4% (n = 18) and between −7.5 and 4.0% (n = 18), respectively (Table 2).

3.6. Stability of plasma sample during storage, matrix effect and recovery

The bench-top stability of cpd-1 in rat plasma was evaluated at room temperature over 24 h using QCs at 3.0 and 650 ng/mL. The measured concentrations of cpd-1 in these QCs were comparable to the nominal values, with accuracy ranging from −2.7 to 12% (Table 3), indicating that cpd-1 was stable for at least 24 h in rat plasma under our storage conditions at room temperature. Freeze–thaw stability of QCs at 0.3 and 750 ng/mL after three cycles were analyzed together with one set of Cs and regular QCs. The measured concentrations of cpd-1 in these QCs were comparable to the nominal values, with accuracy ranging from −11.4 to −7.7% (Table 3). The auto-sampler stability of cpd-1 in rat plasma was evaluated at 5 °C over 51 h using QC samples at 0.3 and 750 ng/mL. The measured concentrations of cpd-1 in these QCs were comparable to the nominal values, with accuracy ranging from −5.9 to 4.0% (Table 3).

The relative recovery was determined in spiked plasma samples (n = 3) at three concentrations: 0.3, 500, and 750 ng/mL by dividing the peak area of cpd-1 sample spiked before extraction by the peak area of an equal concentration of cpd-1 sample in the same matrix spiked after extraction. The overall recovery was 74% (Table 4).

Table 1
Daily variation of calibration parameters in rat plasma.

Run ID	a	b	c	Regression coefficient (R^2)	LLOQ (ng/mL)	ULOQ (ng/mL)
1	−0.000001492	0.08484	0.0009562	0.9991	0.100	1000
2	−0.00001990	0.09944	−0.0009216	0.9986	0.100	1000
3	−0.00001361	0.09552	0.001144	0.9963	0.100	1000
n	3	3	3	3		

Calibration parameters a, b and c of the calibration function $y = ax^2 + bx + c$ and coefficient of determination R^2 on each day of the validation.

Table 2
QCs precision and accuracy data.

Nominal concentration (ng/mL)	0.100	0.300	200	750
Measured concentration (ng/mL)				
Day 1	0.101	0.292	186	708
	0.0905	0.285	183	736
	0.0881	0.305	185	711
	0.106	0.353[a]	179	734
	0.0980	0.297	181	723
Intra-run mean (ng/mL)	0.0967	0.306	183	722
Intra-run %CV	7.7	8.9	1.6	1.8
Intra-run %bias	−3.3	2.0	−8.5	−3.7
Day 2	0.101	0.335	187	753
	0.0832	0.334	180	737
	0.122[b]	0.308	183	762
	0.113	0.332	172	752
	0.120	0.303	183	743
	0.115	0.303	181	737
	0.122[b]	0.315	190	776
Intra-run mean (ng/mL)	0.111	0.319	182	751
Intra-run %CV	12.8	4.6	3.1	1.9
Intra-run %bias	11.0	6.3	−9.0	0.1
Day 3	0.120	0.317	190	759
	0.100	0.321	192	737
	0.0964	0.313	192	739
	0.0957	0.309	189	732
	0.0860	0.290	186	755
	0.0981	0.306	189	745
Intra-run mean (ng/mL)	0.0994	0.309	190	745
Intra-run %CV	11.3	3.5	1.2	1.4
Intra-run %bias	−0.6	3.0	−5.0	−0.7
Inter-run mean (ng/mL)	0.103	0.312	185	741
Inter-run %CV	12.4	5.7	2.8	2.3
Inter-run %bias	3.0	4.0	−7.5	−1.2

[a] >15%bias.
[b] >20%bias.

The matrix effect was determined in spiked plasma samples ($n = 3$) at three concentrations: 0.3, 500, and 750 ng/mL by dividing the concentration of cpd-1 sample in plasma spiked after extraction by an equal concentration of cpd-1 in neat solution [17]. The overall matrix effect factor was >108% suggesting slight MS signal enhancement (Table 4).

3.7. Dilution effect and batch size

The dilution test was determined using a 100-fold QCs dilution with blank plasma prior to extraction and assayed in five replicates along with Cs and QCs in a validation run. As can be seen in Table 5, the measured

Table 3
Room temperature, freeze–thaw and auto-sampler stability of cpd-1 ($n = 3$).

	Room temperature[a]	Freeze–thaw cycles[b]	Auto-sampler[c]
Nominal concentration (ng/mL)	3.0	0.3	0.3
Measured concentration (ng/mL)			
Sample 1	3.28	0.27	0.30
Sample 2	3.37	0.30	0.28
Sample 3	3.39	0.24	0.27
Mean (ng/mL)	3.35	0.27	0.28
%CV	1.7	11.1	6.2
%bias	12	−11.4	−5.9
Nominal concentration (ng/mL)	650	750	750
Measured concentration (ng/mL)			
Sample 1	614	683	750
Sample 2	646	689	772
Sample 3	637	705	824
Mean (ng/mL)	632	692	782
%CV	2.6	1.6	4.9
%bias	−2.7	−7.7	4.0

[a] The room temperature stability was determined after 24 h period of storage.
[b] Stability after three freeze–thaw cycles.
[c] Auto-sampler stability at 5 °C for 51 h.

Table 4
Relative recovery and matrix effects in rat plasma ($n = 3$) at different QCs concentrations.

Concentration (ng/mL)	Relative recovery (%)	Matrix effect (%)	Type of effect
0.3	70.4 ± 9.4	98	2% suppression
500	74.4 ± 1.4	109	9.0% enhancement
750	76.9 ± 6.3	118	18% enhancement
Overall	74	108	8% enhancement

Table 5
Precision and accuracy of dilution QC samples ($n = 5$).

Initial concentration (ng/mL)		2000
Dilution factor		100
	Measured concentration (ng/mL)	Final measured concentration (ng/mL)
Sample 1	19.2	1920
Sample 2	19.2	1920
Sample 3	18.7	1870
Sample 4	19.3	1930
Sample 5	19.3	1930
Mean (ng/mL)		1914
%CV		1.3
%bias		−4.3

concentrations of cpd-1 in these QCs were comparable to the nominal values, with accuracy of −4.3%, demonstrating that samples with higher concentration can be diluted with blank plasma to obtain acceptable data.

3.8. Carryover

In the current assay, tetrahydrofuran/methanol/water (1:1:1, v/v/v) and acetonitrile/water (4:1, v/v) solutions were used to wash syringe and injection port multiple times before and after each injection. Under these washing conditions, no peak was observed at the both retention times of cpd-1 (Fig. 4A) and IS (Fig. 4B) in the SRM chromatogram of a blank extracted sample analyzed right after the injection of cpd-1 at the ULOQ concentration (1000 ng/mL), indicating the absence of carryover.

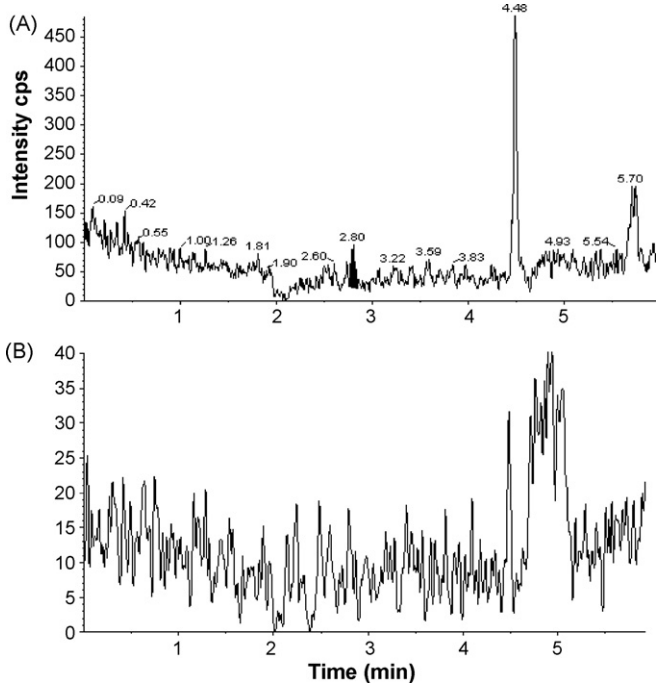

Fig. 4. Representative SRM chromatograms of blank matrix injected right after the ULOQ samples cpd-1 (m/z 291 → 135) (A) and IS (m/z 294 → 138) (B).

3.9. cpd-1 profile in rat plasma and pharmacokinetic data

The concentration time profiles of cpd-1 in plasma after administration of 5 mg/kg intravenously or 25 mg/kg orally are displayed in Fig. 5A and B, respectively. Cpd-1 could be detected in plasma up to 168 h post-dosing where low concentrations of cpd-1 were found by LC–MS/MS. The radioactivity and LC–MS/MS concentration profiles versus time were similar. However, the concentration obtained by LC–MS/MS (only parent cpd-1) was lower than the one found by the radioactivity (parent drug and metabolites), which is consistent with cpd-1 metabolism in rat. Following a 5 mg/kg intravenous dose, the plasma concentration profile versus time of cpd-1 declined rapidly within the first 2 h and was then slowly eliminate from body with a terminal half-life of 45 h. Cpd-1 in rats had a moderate to high plasma steady-state volume of distribution (43 L/kg) (Table 6). Following a 25 mg/kg oral gavage, the C_{max} for cpd-1 in plasma (409 ng/mL) was reached at 1.5 h (first sampling time), indi-

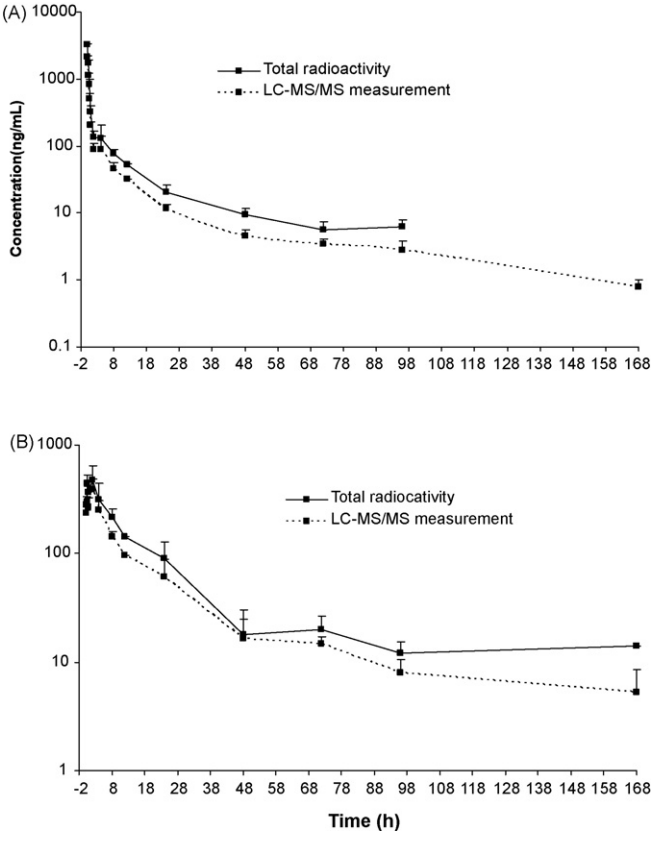

Fig. 5. Concentration time profile of cpd-1 in plasma after measurement by radioactivity or by LC–MS/MS following intravenous (A) or oral administration (B) of [^{14}C]cpd-1.

Table 6
Pharmacokinetic parameters of cpd-1 in plasma.

Parameter	Intravenous (5 mg/kg)		Oral (25 mg/kg)	
	Mean	SD	Mean	SD
C_{max} (ng/mL)	2160	45.8	409	83.4
T_{max} (h)	0.083	0	1.5	0.87
AUC_{0-last} (ng h/mL)	2410	344	5620	430
$AUC_{0-\infty}$ (ng h/mL)	2460	362	6360	894
$t_{1/2}$ (h)	45	4.5	84	32
V_{ss} (mL/kg)	42,500	1740		
CL (mL/h/kg)	2060	316		
F (%)		51.7	31.4	

The first sampling time was 0.083 h for *i.v.* and 0.25 h for *p.o.*

cating a rapid absorption rate cpd-1. The rat bioavailability was 52% and the apparent terminal half-life was 84 h (Table 6).

4. Conclusions

A sensitive, selective and rapid LC–MS/MS method using protein precipitation and sample analysis on C_{16} column was developed for the quantification of NAs in rat plasma. The simplicity of the current assay would make its implementation highly useful for supporting NAs selection within the frame of the drug discovery program. The assay has been demonstrated to be extremely rugged and has been successfully used for the routine analysis of plasma samples to generate PK parameters of NAs and their derivatives in the early stage of compound development.

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