Development and validation of a LC-MS/MS method for the quantification of the checkpoint kinase 1 (CHK1) inhibitor SRA737 in human plasma

Running title: Analysis of SRA737 in human plasma

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Development and validation of a LC-MS/MS method for the quantification of the checkpoint kinase 1 (CHK1) inhibitor SRA737 in human plasma

Abstract

Background: SRA737 is an orally active small molecule inhibitor of Checkpoint kinase 1 being investigated in an oncology setting. A HPLC-MS/MS method for quantifying plasma concentrations of SRA737 was validated.

Methods & results: Sample preparation involved protein precipitation with acetonitrile following addition of $^{13}C^{15}N$ deuterated SRA737 as internal standard. A rapid and selective method was fully validated across a range of 5-20,000 ng/mL, exhibiting good sensitivity, overall precision (expressed as CV) ≤8.0% and accuracy 96-102%. Consistently high recovery was observed, with no matrix effect and a lower limit of quantitation of 5 ng/mL.

Conclusion: A novel method for analysing SRA737 in human plasma has been validated and is now being utilised for quantification of SRA737 in a phase I trial.

Keywords: CCT245737, SRA737, CHK1, LC-MS/MS, validation study, pharmacokinetics, cancer

Introduction

In an oncology setting the use of chemotherapy or radiotherapy is frequently associated with DNA damage [1]. In response to DNA damage, cells activate the DNA damage response (DDR), involving multiple signalling pathways such as cell cycle checkpoints, DNA repair, transcriptional programs and apoptosis. Cells maintain genomic integrity via the DNA damage response (DDR) which is critical for survival and proliferation. Intrinsic genomic instability and an over-reliance on the DDR machinery is a hallmark feature of tumour development prompting the development of agents targeting DDR signalling pathways, particularly checkpoint kinase 1 (CHK1), which plays a key role in the DNA-damage checkpoint signal transduction pathway [2]. CHK1 has been shown to play a role in the disruption or halting of DNA replication to facilitate DNA repair following treatment with a variety of chemotherapeutics in mammalian cells [3, 4]. CHK1 inhibitors are predicted to prevent cells entering cell cycle arrest, thereby enhancing the activity of genotoxic agents such as gemcitabine and cisplatin. Chk1 inhibitors are also predicted to demonstrate synthetic lethality as monotherapy in tumours with certain genetic profiles.

SRA737 (formerly known as CCT245737) is an orally active small molecule inhibitor of CHK1 discovered at the Cancer Therapeutics Unit, Institute of Cancer Research, and developed for use in an oncology setting [5]. Inhibition of CHK1 function by SRA737 can result in substantial increases in the sensitivity of tumour cells to a variety of anticancer drugs [6, 7]. In this respect, SRA737 was shown to improve gemcitabine and SN38 antitumor activity without increasing toxicity in a human tumour xenograft model [7]. Following the generation of promising preclinical data, SRA737 is currently being tested in a Phase I clinical trial setting.

To support the Phase I study of SRA737, a robust method for the quantification of SRA737 in human plasma is required for the generation of pharmacokinetic data. Thus far, no validated method for the quantification of SRA737 has been published. The current work describes the development and validation of a high performance liquid chromatography tandem mass spectrometry (LC–MS/MS) assay for the quantification of SRA737 in human plasma. The assay has been validated according to European Medicines Agency (EMA) and US Food and Drug Administration (FDA) guidelines for bioanalytical method validation [8, 9] and successfully

applied to support a pharmacokinetic study in advanced cancer patients in a Phase I clinical trial setting.

Experimental

Standards & chemicals

Analytical standards of SRA737 (MW 379.34 g/mol) and labelled SRA737 ($^{13}C^{15}N$ deuterated SRA737; MW 383.35 g/mol) were provided by Cancer Research UK (Figure 1). HPLC grade acetonitrile, acetic acid and ammonium hydroxide were purchased from Fisher Scientific (Leics, UK). Control human plasma with sodium citrate, used to prepare daily standard calibration curves and quality control samples (QCs), was obtained from the Blood Transfusion Centre (Newcastle, UK).

Standard solutions

Two separate stock solutions of SRA737 for standards and QCs were prepared in dimethylformamide (DMF) at a concentration of 1 mg/mL. A stock solution of $^{13}C^{15}N$ deuterated SRA737 (Internal Standard; IS) was prepared at 1 mg/mL in DMF. Stock solutions were diluted serially in DMF to obtain working solutions, with final SRA737 concentrations of 0.4, 1, 2, 20, 100, 200 and 400 µg/mL for standards and 0.6, 60 and 300 µg/mL for working solutions of QCs. These solutions were used to prepare calibration curve standards and QC samples in control human plasma. The IS working solution was prepared at a concentration of 1 µg/mL by diluting the stock solution with DMF. All solutions were stored at -20 °C prior to use.

A ten-point calibration curve was utilised, with standard calibration samples prepared by adding 10 µL of the working standard solutions to plasma (190 µL), to produce final SRA737 concentrations of 5, 10, 20, 50, 100, 150, 1000, 5000, 10000 and 20000 ng/mL. Each run

included a blank sample (plasma control processed without IS) and a zero blank sample (plasma control processed with IS). The QC samples were prepared by adding 10 µL of each working QC solution to human plasma control (190 µL) to obtain SRA737 concentrations of 30, 3000 and 15000 ng/mL.

Processing samples

Plasma aliquots (20 µL) from study samples, standards or QC samples were vortex mixed with 10 µL (10 ng) of IS working solution and 100 µL of acetonitrile and samples were centrifuged at 4000 g for 5 minutes at room temperature. The supernatant obtained (100 µL) was transferred to an Eppendorf tube and 100 µL of mobile phase A was added. Following vortex mixing, samples were transferred to autosampler vials and 3 µL volumes were routinely injected onto the HPLC-MS/MS system.

Chromatography conditions

A Prominence series HPLC system was utilised, consisting of a SIL-20AC XR autosampler, two LC-20AD XR pumps, a CBM-20A communications bus module and a CTO-20AC column oven (Shimadzu, Milton Keynes, Buckinghamshire, UK). A Phenomenex Kinetex C18 column (2.6 µm, 50.0 x 4.6 mm) with a Phenomenex Security guard containing a C18 cartridge (4 x 2mm) was utilised for sample separation. Mobile phase A (MP A) consisted of 10 mM ammonium acetate + 0.5% ammonia (v/v) and MP B was acetonitrile. The HPLC system was set at a constant flow rate of 0.5 mL/min and run under gradient conditions: step 1 - 95% MP A for 1 min; step 2 - 95% MP A to 5% over 3 min; step 3 - constant for 1 min; step 4 - 5% MP A to initial conditions over 1 min; step 5 - reconditioning for 4 min.

Mass spectrometry conditions

An API 4000 triple quadrupole mass spectrometer from SCIEX (Foster City, CA, USA) was utilised in the current assay. MS parameters were optimised through the infusion of standard solutions (10 ng/mL) of SRA737 and IS at a flow rate of 0.5 mL/min. Positive ion mode was used to obtain the mass spectra (MS1) and the product ion spectra (MS2). Fragment selection during compound tuning was based on an initial fragmentation screen which produced four prominent fragments. These four fragments were further optimised for collision energy and collision cell exit potential and the best three selected for flow injection analysis and background testing. After testing in blank matrix under final chromatographic conditions, the best performing fragment by signal-to-noise comparison was chosen as the final transition. The instrument incorporated a Turbo Ion Spray source operated at 650°C, with voltage of 5500 V. Biological samples were analysed with electrospray ionization (ESI), using zero air as the nebulizer gas (206.8 kPa) and as heater gas (482.6 kPa). Nitrogen was employed as curtain gas (206.8 kPa) and as collision gas at 34.5 kPa (CAD). The declustering potential (DP) was optimized and set to 71V for SRA737 and 61V for the $^{13}C^{15}N$ deuterated SRA737. Quantification was carried out in Selected Reaction Monitoring (SRM) mode following the transitions m/z 379.872 → 360.200 for SRA737 and m/z 384.086 → 324.200 for the IS. Data processing was carried out with Analyst 1.6.2 software package (SCIEX).

Method validation

Validation of the method was carried out according to EMA and FDA bioanalytical method validation guidance documents [8, 9]. Parameters validated included selectivity, anticoagulant comparison, matrix effect, recovery, lower limit of quantification (LLOQ),

linearity and range, dilutional integrity, carry-over effect, intra/inter-assay precision, accuracy and stability.

Selectivity

The selectivity of the method was assessed by analysing 6 independent sources of blank plasma. Any response with similar retention time to the analyte was required to be ≤ 20% of the response for the lowest concentration included in the standard curve. Any response with a similar retention time to the IS was required to be ≤ 5% of the response for the IS peak [8, 9]. Experiments to investigate the interference of potentially co-administered drugs were not carried out as part of the assay method validation.

Anticoagulant comparison

The potential effects of different anticoagulants were determined by analysing 3 replicates at LQC and HQC concentrations of SRA737, which were prepared using blank plasma obtained through the use of 3 different anticoagulants: sodium citrate, potassium EDTA and lithium heparin. The coefficient of variation (CV) was required to be within 15% and accuracy within 85-115% [8, 9].

Matrix effect

Six independent sources of blank matrix for SRA737 at low and high QC concentrations and for the IS were utilised to calculate the matrix factor (MF) for each analyte, i.e. the ratio of the peak area of the analyte added to a pre-extracted sample to the peak area of an equal amount of analyte in solvent. The IS normalised MF was calculated by dividing the MF of SRA737 by the MF of IS. The CV of the IS-normalised MF was required to be within 15% [8, 9].

197

198 *Recovery*

199 Percentage extraction data were obtained using 3 QC concentrations (30, 3000 and 15000
200 ng/mL) for SRA737 and at 500 ng/mL for the IS in plasma samples processed in triplicate. The
201 peak area of SRA737 extracted from plasma samples was compared to the peak area in
202 absence of matrix (true concentration of the analyte in solvent) to calculate the absolute
203 recovery. The CV was required to be within 15% [8, 9].

204

205 *Limit of quantification*

206 The LLOQ for the assay was defined as the concentration of the lowest standard with precision
207 $\leq$ 20% and accuracy within 80-120% of the nominal value, with a signal-to-noise ratio ≥10.
208 The defined LLOQ was assessed by preparing five plasma samples with SRA737 at a final
209 concentration of 5.0 ng/mL, with the experiment repeated on 4 separate days [8, 9].

210

211 *Linearity and Range*

212 Calibration curve linearity was investigated over seven working days, with the linear range
213 determined over one working day through the preparation of samples <50% of the lowest
214 concentration included in the standard curve and >150% of the upper limit of quantification
215 (ULOQ). The ratio of the HPLC-MS/MS peak area for SRA737 to IS was calculated for each
216 standard concentration and plotted against the nominal concentration of drug in the sample.
217 Standard curve linearity was assessed by regression analysis and goodness of fit using
218 Pearson's determination coefficient R^2 and through comparison of true and back-calculated
219 concentrations of calibration standards. Back-calculated values were required to be within
220 85-115% of the theoretical concentration (80-120% at the lowest concentration included in

the standard curve), and at least 75% of the standards were required to meet these criteria, including the lowest and the highest calibrators [8, 9].

Dilution integrity and carryover

A 50 µg/mL solution of SRA737 was made in plasma and diluted 1 in 10 with control plasma to generate a 5000 ng/mL standard. Samples were prepared in 5 replicates. Accuracy and precision were required to be within ±15% [8]. Carryover of SRA737 and IS were evaluated by placing a blank sample directly after the highest calibration standard. Carryover sample analyte response was required to be ≤20% of the response observed for the lowest concentration on the standard curve. The IS response in the carryover sample was required to be ≤5% of the response for the control matrix + IS [8, 9].

Intra/Inter-assay precision and accuracy

Intra-day precision and accuracy were investigated using five replicates per QC concentration, with data from four separate experiments carried out on different days generated to assess inter-day precision and accuracy. The precision of the method at each concentration was reported as the CV value, expressing the standard deviation as a percentage of the mean calculated concentration; accuracy was determined by expressing mean calculated concentrations as a percentage of the nominal concentration. Concentrations determined for QC samples in each run were required to be within 15% of the nominal value, with the exception of the LLOQ which should be within 20% [8, 9].

Stability

SRA737 stability in plasma was assessed by analysing low and high QC samples in triplicate following storage under various different conditions. Short-term stability was investigated using QC samples both unextracted and extracted (autosampler stability) from the plasma matrix after 7 days storage at 4°C, with bench-top stability at room temperature calculated over 4 h. Freeze–thaw stability was determined for three cycles over a range of -20°C and room temperature. Long-term stability was investigated using QC samples stored for 8 months at -20°C. SRA737 QC samples were analysed against a calibration curve generated from freshly spiked standards, with the concentrations determined compared to the nominal concentrations. The mean obtained QC concentration was required to be within ±15% of the nominal concentration [8, 9].

Application of method to clinical sample analysis

The method was used to quantify SRA737 plasma concentrations in a patient with advanced cancer treated on the ongoing Phase I clinical trial of SRA737 administered as single agent monotherapy (EudraCT number 2015-004486-86). Blood samples for pharmacokinetic analysis were obtained prior to administration of a single oral dose of 40 mg SRA737 and at 0.5, 1, 2, 4, 6, 8, 12 and 24 h post-administration. Blood samples (2 mL) were collected into EDTA tubes and centrifuged at 1200 g for 5 min at 4°C. Plasma was separated and frozen at -20°C prior to analysis as described above.

Results & discussion

HPLC-MS/MS

Using an ESI source in positive ion mode, SRA737 formed mainly a molecular ion M^+ at m/z 379.872, while $^{13}C^{15}N$ deuterated SRA737 (IS) formed a protonated molecule $[M+H]^+$ at m/z 384.086. These precursor ions passed through the first quadrupole into the collision cell and the collision energy (CE) was optimized to obtain a high signal for the product ions generated. After fragmentation, the characteristic products were monitored in the third quadrupole at m/z 320.2 (35 eV), 360.2 (25 eV) and 255.0 (39 eV) for SRA737 and at m/z 324.2 (33 eV) and 259.2 (39 eV) for the IS. The fragmentation patterns are presented in Figure 2; SRA737 and IS were quantified using the transitions *m/z* 379.872 → 360.200 (Figure 2A) and *m/z* 384.086 → 324.200 (Figure 2B). Figure 3 represents typical SRM chromatograms, using the same SRA737 and IS transitions as above. Figure 3A shows an extracted blank plasma sample; Figure 3B shows an extracted blank plasma sample with IS added; Figure 3C represents an extracted plasma sample at the LLOQ (5 ng/mL) with IS. The elution of the analytes was efficient and selective, SRA737 and IS being eluted at ~4 min. No interfering peaks were observed at these retention times and the peaks were completely resolved from the plasma matrix with good peak shape observed. The specificity of the method was confirmed by analysing six independent sources of blank human plasma.

Method validation

Selectivity

Selectivity was evaluated on 6 different batches of human plasma, including haemolysed plasma. The method shown to be selective with an absence of interfering components.

Response with similar retention time was <7 % of the LOQ and <1 % for the IS in six batches of plasma evaluated.

Anticoagulant comparison and matrix effect

The potential effect of different anticoagulants was determined by analysing 3 replicates at LQC and HQC concentrations of SRA737, prepared using blank plasma obtained through the use of potassium EDTA and lithium heparin, as compared to control citrate plasma. No effect of different anticoagulants was observed, with a calculated CV ≤ 4.8 % and accuracy within the range 97-100 %, indicating that plasma obtained from blood samples collected with any of these commonly used anticoagulants could be utilised to generate accurate results. The matrix effect was evaluated on 6 different batches of human plasma, including haemolysed plasma at LQC and HQC concentrations. There were no significant differences between the six lots evaluated by assessment of IS-corrected matrix factor, with calculated values of 1.02 ± 0.03 for LQC (CV 3.4%) and 0.96 ± 0.02 for HQC (CV 2.1 %).

Recovery and limit of quantification

Recovery was determined in triplicate using three QC concentrations through comparison of peak areas of spiked plasma samples following extraction, with peak areas obtained from direct injection of SRA737 standards in mobile phase. Recovery percentages for SRA737 were 114%, 95.7% and 93.7% at concentrations of 30, 3000 and 15000 ng/mL, respectively, with a recovery of 102% observed for the IS. There were no significant variations (1.8-8.3%) for the peak areas of SRA737 and IS and all data generated were in the anticipated and acceptable range. The LLOQ concentration in plasma was defined to be 5.0 ng/mL, with precision and accuracy of 5.4% and 102.9%, respectively determined by preparing five plasma samples with

CCT245737 at a final concentration of 5.0 ng/mL, with the experiment repeated on 4 separate days, at this concentration of SRA737 (see Table 2). For LLOQ and LQC concentrations the volume of injection was increased from 3 µL to 10 µL. All results are expressed as a ratio of the peak area of SRA737 to IS and therefore the overall data generated are unaffected by sample volume.

Linearity and Range

Linearity was investigated over ten concentrations of SRA737 (range 5-20000 ng/mL), with a linear correlation of ≥0.997 calculated from 7 separate experiments. The calibration curve was typically described by the linear equation $y = 1.75x + 0.003$, with $1/y^2$ weighting. The weighting of $1/y^2$ gave the best linear response, with consistent % relative error values across the standard curve concentration range and correlation coefficients with excellent reproducibility. Table 1 shows linearity and range data over a SRA737 concentration range of 5-20000 ng/mL.

Dilution integrity and carryover

A 50 µg/mL spiked sample was generated in plasma and diluted 1 in 10 in plasma to a concentration of 5000 ng/mL, with an accuracy of 91% observed. Carry-over effects were negated by injecting 2 mobile phase samples and 2 extracted blank plasma samples after the injection of ULOQ samples or high concentration samples.

Intra/Inter-assay precision, accuracy and stability

The intra-assay study showed precision ≤5.6 % and accuracy ranging from 96.5 - 100 % (*n* = 5). The inter-assay study over four days showed precision ≤7.9 % and accuracy 96.5 - 106 %

as shown in Table 2. SRA737 stability in plasma was determined by analysing triplicate QC samples at low and high concentrations. SRA737 was stable in plasma for at least 4 h at room temperature and for 7 days at 4°C both before and after drug extraction. SRA737 was stable in plasma at -20°C after 8 months of storage and over 3 freeze-thaw cycles. Standard working solutions of SRA737 and IS, prepared in DMF and stored at -20°C, were stable for at least 2 months. Table 3 provides a summary of the stability data generated as part of the assay validation. In addition to these stability experiments formalised in the method validation plan, experiments were also carried out to investigate the impact of storing whole blood samples from patients who have received SRA737, for defined time periods ahead of plasma separation and storage. Results indicated that storage of whole blood samples for 8 h or 24 h at either room temperature or 4°C had no effect on the concentration of SRA737 determined in plasma, with mean values varying <5% from data obtained when whole blood samples were centrifuged immediately following collection.

Clinical sample analysis

Analysis of plasma samples obtained from a patient receiving a single oral dose of 40 mg SRA737 indicated that the assay could successfully be utilised to quantify SRA737 in clinical trial samples. Figure 3D shows an SRM chromatogram obtained from an extracted plasma sample collected from a patient receiving SRA737 and Figure 4 shows the plasma concentration-versus-time curve for SRA737 at a dose of 40 mg. Quantifiable drug levels were measured over a 24h period following drug administration, with a Cmax of 95 ng/mL observed at a Tmax of 1 h.

Conclusions & future perspective

The bioanalytical method described has been validated for the quantitative measurement of the CHK1 inhibitor SRA737 in human plasma obtained from patients currently participating in early phase clinical trials with this promising drug candidate. The method utilises small plasma volumes, is rapid, highly sensitive, precise and accurate. The observed limit of quantification clearly facilitates the determination of SRA737 concentrations in clinical samples, even at low doses administered during the early patient cohorts recruited to the ongoing clinical trials.

Experiments to investigate the interference of potentially co-administered drugs were not carried out as part of the assay method validation as it was felt that it was unlikely that co-administered drugs with contrasting chemical structures would interfere with the assay and due to the number of potentially co-administered drugs being prescribed in late stage cancer patients participating in a phase I clinical trial. However, further experiments to investigate potential interactions with specific co-administered anticancer drugs may be warranted for future drug combination studies. It should also be highlighted that as SRA737 is at an early stage of clinical development, incurred sample reanalysis experiments have not yet been carried out. The FDA guidance and EMEA guideline on Bioanalytical method validation stipulate that 7-10% of the samples should be analysed around the Cmax and in the elimination phase. Further information will be gathered on these aspects prior to initiating incurred sample reanalysis studies.

381 The assay is now being utilised to generate novel data concerning the pharmacokinetics of
382 SRA737, facilitating investigations into the importance of systemic drug exposure to this agent
383 in determining clinical response and toxicity in cancer patients.
384

Executive Summary

Background

• a HPLC-MS/MS method was developed to quantify the novel anticancer drug SRA737 in human plasma obtained from patients participating in an early phase clinical trial.

Experimental

• The method utilises small samples volumes (20µL), involving protein precipitation with acetonitrile and use of $^{13}C^{15}N$ deuterated SRA737 as internal standard.

Results and discussion

• A rapid and selective assay was developed according to EMA and FDA guidelines for bioanalytical method validation, covering a range of 5-20,000 ng/mL as appropriate for the analysis of drug levels in patient samples.

• The method is being utilised successfully to quantify SRA737 in clinical samples obtained from patients participating in an ongoing phase I trial.

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427 **TABLES**

428 **Table 1.** Inter-day linearity, accuracy and precision of calibration curves of SRA737 in human plasma

429

	SRA737 plasma concentrations (ng/mL)									
Day	**5.0**	**10.0**	**20.0**	**50.0**	**100**	**500**	**1000**	**5000**	**10000**	**20000**
1	4.9	10.5	19.7	51.7	99	547	988	5049	9794	18865
2	5.2	9.8	18.5	51.6	103	509	1068	5174	9300	20160
3	4.9	10.1	19.9	51.6	104	554	1030	5158	9302	18018
4	5.0	9.7	21.6	51.9	101	532	1023	4933	9900	18085
5	5.0	9.7	20.6	51.9	107	485	998	5367	10053	18116
6	5.0	10.0	19.1	53.5	102	491	979	5504	9143	20633
7	5.0	9.9	19.8	48.9	105	515	1037	5414	9286	19504
Mean (n=4)	5.0	10.0	19.9	52	103	519	1018	5229	9540	19054
SD	0.1	0.3	1.0	1.4	2.6	26.6	31.2	207.0	363.7	1068.4
Accuracy (%)	100.1	99.7	99.4	103.2	103.1	103.8	101.8	104.6	95.4	95.3
Precision (%)	1.8	2.9	5.1	2.7	2.5	5.1	3.1	4.0	3.8	5.6

SD: Standard deviation

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435 **Table 2.** Intra/Inter-day precision and accuracy of the method for the analysis of SRA737 in human
436 plasma (LLOQ, LQC, MQC and HQC)

437

Sample	Day	SRA737 concentration (ng/mL)		Accuracy (%)	Precision (%)	SRA737 concentration (ng/mL)		Accuracy (%)	Precision (%)	RE (%)
		Mean	SD			Mean	SD			
LLOQ	1	5.46	0.15	109.2	3.27					
	2	5.02	0.13	100.4	2.89					
	3	5.04	0.27	100.8	5.02					
	4	5.06	0.30	101.2	6.03					
	1-4					5.15	0.28	102.9	5.4	-2.9
LQC	1	30.6	2.08	102	6.80					
	2	31.7	2.49	106	7.87					
	3	29.3	0.57	97.5	1.94					
	4	31.2	2.21	104	7.06					
	1-4					30.7	1.05	102	3.41	2.33
MQC	1	2933	104	97.8	3.55					
	2	3020	113	101	3.73					
	3	2896	161	96.5	5.57					
	4	3033	92.9	101	3.06					
	1-4					2971	66.7	99.0	2.24	-0.98
HQC	1	15000	700	100	4.67					
	2	15300	436	102	2.85					
	3	15040	577	100	3.84					
	4	15500	100	103	0.65					
	1-4					15210	235	101	1.54	1.40

438

439 *NA: Not applicable; SD: Standard Deviation; RE: Relative Error*

440

441 **Table 3.** Short and long-term stability data for SRA737 in various different matrices

442

Temp	Matrix	Time	LQC results from 3 replicates				HQC results from 3 replicates			
			Mean	SD	Accuracy (%)	CV (%)	Mean	SD	Accuracy (%)	CV (%)
RT	plasma	4 hrs	29.3	1.11	97.7	3.80	14500	557	96.7	3.84
4°C	plasma	7 days	30.8	3.50	103	11.4	13967	115	93.1	0.83
4°C	MP	7 days	30.6	1.89	102	6.17	15400	265	103	1.72
-20°C	plasma	1 FTC	29.2	1.37	97.2	4.68	15967	874	106	5.47
-20°C	plasma	2 FTC	29.8	2.31	99.4	7.74	14700	700	98.0	4.76
-20°C	plasma	3 FTC	29.7	0.68	98.9	2.29	15333	473	102	3.08
-20°C	DMF	2 months	31.0	1.07	103	3.45	14800	436	98.7	2.95
-20°C	plasma	2 months	29.5	2.11	98.4	7.14	14550	495	97.0	3.40
-20°C	plasma	8 months	29.2	4.03	97.3	13.8	14233	1101	94.9	7.74

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444 *RT: Room temperature; MP: Mobile phase; FTC: Freeze thaw cycle; DMF: Dimethylformamide*

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448 **Figure Legends**

449

450 **Figure 1.** Chemical structures of (A) SRA737 (Molecular Weight: 379.34) and (B) labelled
451 SRA737 ([$^{13}C^{15}N$] deuterated SRA737) (Molecular Weight: 383.35)

452

453 **Figure 2.** MS and MS/MS mass spectra of (A) SRA737 and (B) IS ([$^{13}C^{15}N$] deuterated SRA737).

454

455 **Figure 3.** (A) SRM chromatograms of a human blank plasma sample (volume of injection 3 µl);
456 (B) SRM chromatograms of a human blank plasma sample with IS (volume of injection 3 µl);
457 (C) signal-to-noise ratio of SRA737 at the LLOQ (volume of injection 10 µl); (D) SRM
458 chromatograms showing SRA737 and the IS of a plasma sample collected from a patient
459 receiving a single oral dose of 40mg SRA737 (volume of injection 3 µl).

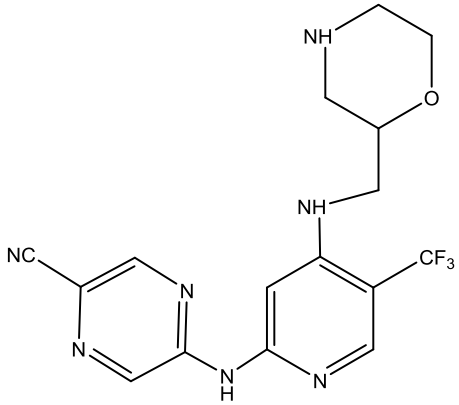
460

461 **Figure 4.** Plasma concentration-versus-time profile of SRA737 in a patient following a single
462 oral administration of 40mg SRA737.

463

Figure 1

A



B

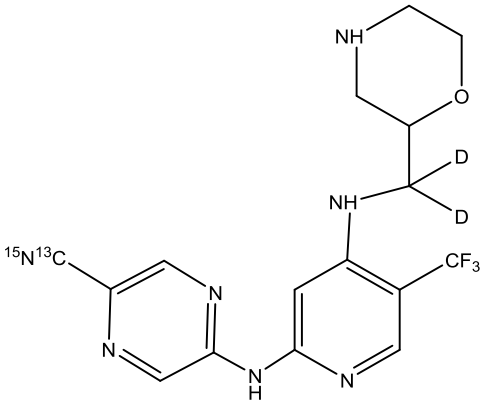
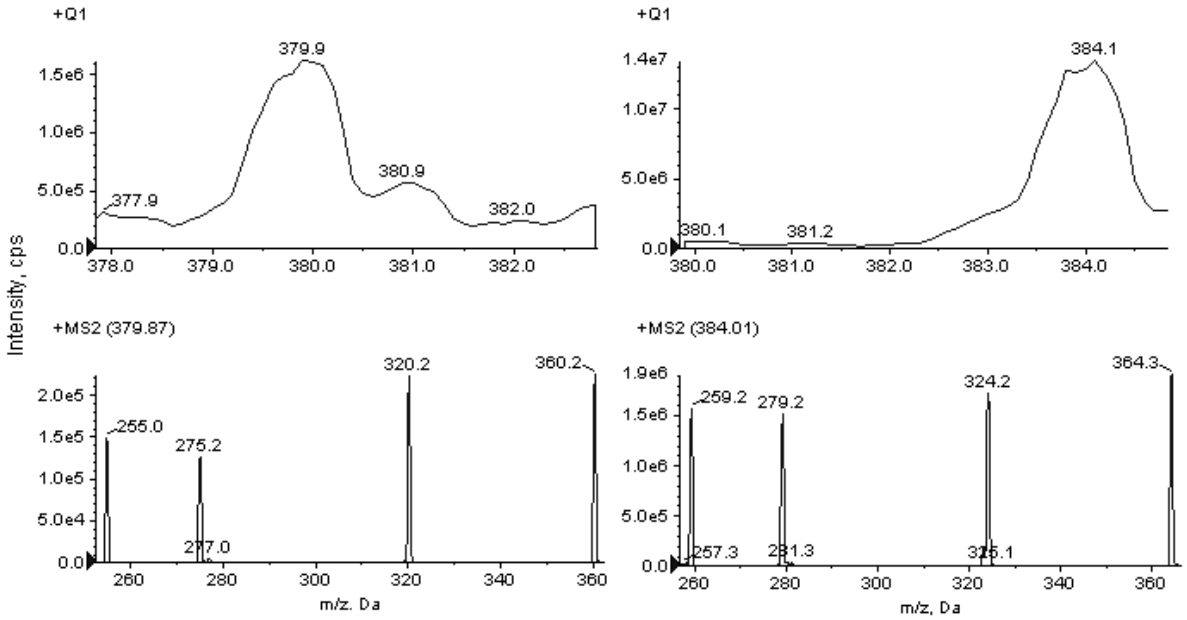


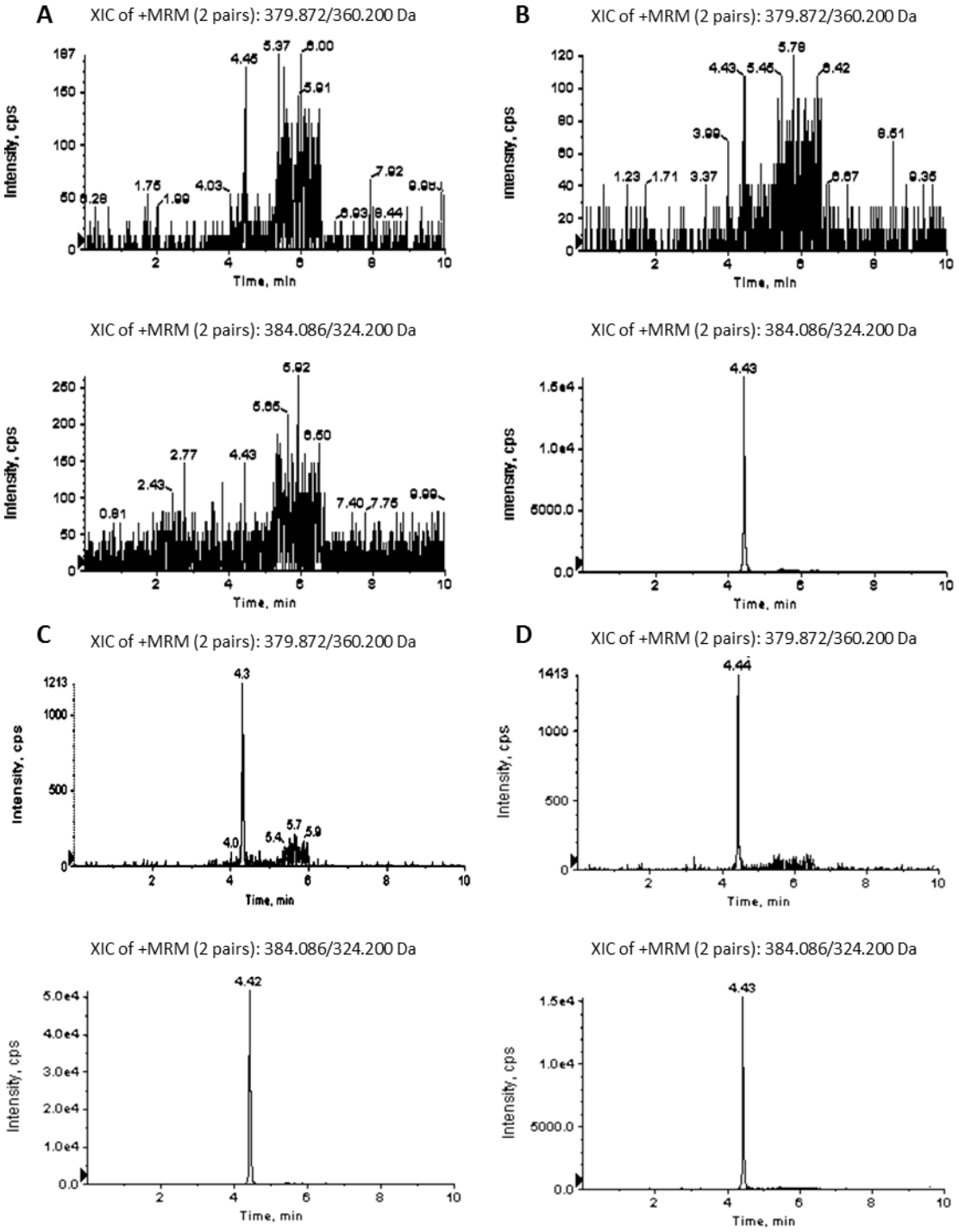
Figure 2

A **B**


+Q1
379.9
1.5e6
1.0e6
5.0e5
0.0
377.9
380.9
382.0
378.0
379.0
380.0
381.0
382.0
Intensity, cps
+MS2 (379.87)
320.2
360.2
2.0e5
1.5e5
1.0e5
5.0e4
255.0
275.2
277.0
260
280
300
320
340
360
m/z, Da
+Q1
384.1
1.4e7
1.0e7
5.0e6
380.1
381.2
380.0
381.0
382.0
383.0
384.0
+MS2 (384.01)
364.3
1.9e6
1.5e6
1.0e6
5.0e5
259.2
279.2
324.2
257.3
281.3
315.1
260
280
300
320
340
360
m/z, Da

476 **Figure 3**

477



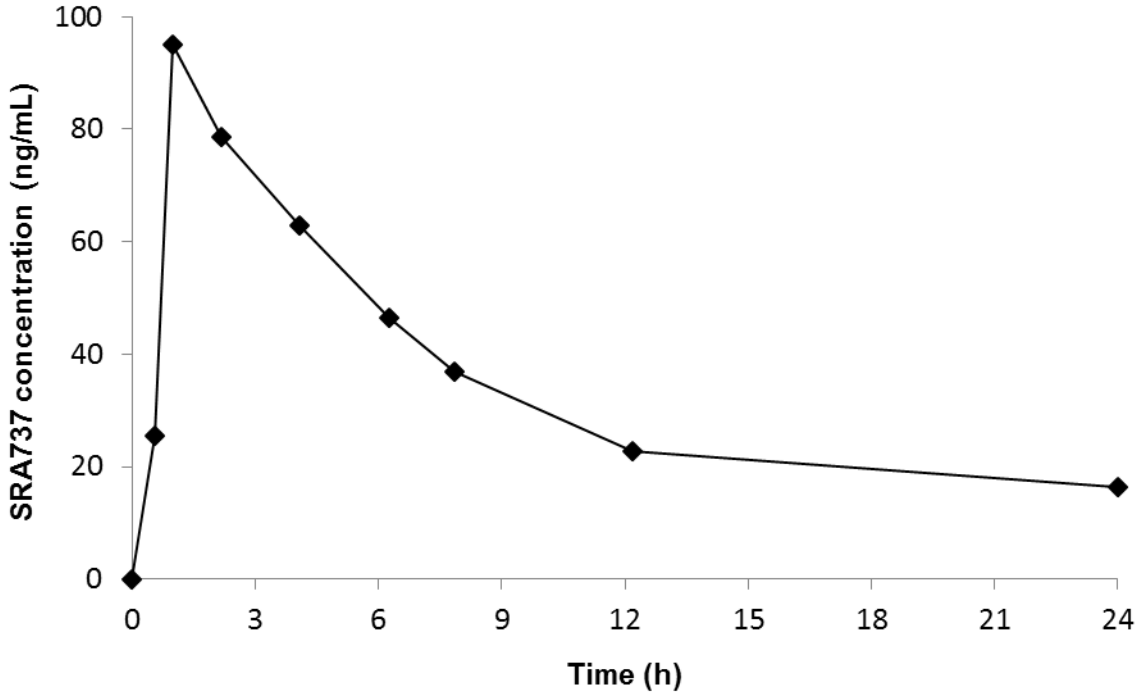
478

479

480

481 **Figure 4**

482



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