

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

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4 Running title: Analysis of SRA737 in human plasma
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33 Dr Raynaud and Dr Banerji are employees of the Institute of Cancer Research (ICR), which has
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43 **Ethical conduct of research**

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

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

54

55 **Abstract**

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

59 **Methods & results:** Sample preparation involved protein precipitation with acetonitrile
60 following addition of ¹³C¹⁵N deuterated SRA737 as internal standard. A rapid and selective
61 method was fully validated across a range of 5-20,000 ng/mL, exhibiting good sensitivity,
62 overall precision (expressed as CV) ≤8.0% and accuracy 96-102%. Consistently high recovery
63 was observed, with no matrix effect and a lower limit of quantitation of 5 ng/mL.

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

66

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

69

70 **Introduction**

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

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

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

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

101

102

103 **Experimental**

104 *Standards & chemicals*

105 Analytical standards of SRA737 (MW 379.34 g/mol) and labelled SRA737 (¹³C¹⁵N deuterated
106 SRA737; MW 383.35 g/mol) were provided by Cancer Research UK (Figure 1). HPLC grade
107 acetonitrile, acetic acid and ammonium hydroxide were purchased from Fisher Scientific
108 (Leics, UK). Control human plasma with sodium citrate, used to prepare daily standard
109 calibration curves and quality control samples (QCs), was obtained from the Blood
110 Transfusion Centre (Newcastle, UK).

111

112 *Standard solutions*

113 Two separate stock solutions of SRA737 for standards and QCs were prepared in
114 dimethylformamide (DMF) at a concentration of 1 mg/mL. A stock solution of ¹³C¹⁵N
115 deuterated SRA737 (Internal Standard; IS) was prepared at 1 mg/mL in DMF. Stock solutions
116 were diluted serially in DMF to obtain working solutions, with final SRA737 concentrations of
117 0.4, 1, 2, 20, 100, 200 and 400 µg/mL for standards and 0.6, 60 and 300 µg/mL for working
118 solutions of QCs. These solutions were used to prepare calibration curve standards and QC
119 samples in control human plasma. The IS working solution was prepared at a concentration
120 of 1 µg/mL by diluting the stock solution with DMF. All solutions were stored at -20 °C prior
121 to use.

122

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

126 included a blank sample (plasma control processed without IS) and a zero blank sample
127 (plasma control processed with IS). The QC samples were prepared by adding 10 µL of each
128 working QC solution to human plasma control (190 µL) to obtain SRA737 concentrations of
129 30, 3000 and 15000 ng/mL.

130

131 *Processing samples*

132 Plasma aliquots (20 µL) from study samples, standards or QC samples were vortex mixed
133 with 10 µL (10 ng) of IS working solution and 100 µL of acetonitrile and samples were
134 centrifuged at 4000 g for 5 minutes at room temperature. The supernatant obtained (100
135 µL) was transferred to an Eppendorf tube and 100 µL of mobile phase A was added.

136 Following vortex mixing, samples were transferred to autosampler vials and 3 µL volumes
137 were routinely injected onto the HPLC-MS/MS system.

138

139 *Chromatography conditions*

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

149

150 *Mass spectrometry conditions*

151 An API 4000 triple quadrupole mass spectrometer from SCIEX (Foster City, CA, USA) was
152 utilised in the current assay. MS parameters were optimised through the infusion of standard
153 solutions (10 ng/mL) of SRA737 and IS at a flow rate of 0.5 mL/min. Positive ion mode was
154 used to obtain the mass spectra (MS1) and the product ion spectra (MS2). Fragment selection
155 during compound tuning was based on an initial fragmentation screen which produced four
156 prominent fragments. These four fragments were further optimised for collision energy and
157 collision cell exit potential and the best three selected for flow injection analysis and
158 background testing. After testing in blank matrix under final chromatographic conditions, the
159 best performing fragment by signal-to-noise comparison was chosen as the final transition.
160 The instrument incorporated a Turbo Ion Spray source operated at 650°C, with voltage of
161 5500 V. Biological samples were analysed with electrospray ionization (ESI), using zero air as
162 the nebulizer gas (206.8 kPa) and as heater gas (482.6 kPa). Nitrogen was employed as curtain
163 gas (206.8 kPa) and as collision gas at 34.5 kPa (CAD). The declustering potential (DP) was
164 optimized and set to 71V for SRA737 and 61V for the ¹³C¹⁵N deuterated SRA737.
165 Quantification was carried out in Selected Reaction Monitoring (SRM) mode following the
166 transitions m/z 379.872 → 360.200 for SRA737 and m/z 384.086 → 324.200 for the IS. Data
167 processing was carried out with Analyst 1.6.2 software package (SCIEX).

168

169 *Method validation*

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

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

175

176 *Selectivity*

177 The selectivity of the method was assessed by analysing 6 independent sources of blank
178 plasma. Any response with similar retention time to the analyte was required to be $\leq 20\%$ of
179 the response for the lowest concentration included in the standard curve. Any response with
180 a similar retention time to the IS was required to be $\leq 5\%$ of the response for the IS peak [8,
181 9]. Experiments to investigate the interference of potentially co-administered drugs were not
182 carried out as part of the assay method validation.

183

184 *Anticoagulant comparison*

185 The potential effects of different anticoagulants were determined by analysing 3 replicates at
186 LQC and HQC concentrations of SRA737, which were prepared using blank plasma obtained
187 through the use of 3 different anticoagulants: sodium citrate, potassium EDTA and lithium
188 heparin. The coefficient of variation (CV) was required to be within 15% and accuracy within
189 85-115% [8, 9].

190

191 *Matrix effect*

192 Six independent sources of blank matrix for SRA737 at low and high QC concentrations and
193 for the IS were utilised to calculate the matrix factor (MF) for each analyte, i.e. the ratio of
194 the peak area of the analyte added to a pre-extracted sample to the peak area of an equal
195 amount of analyte in solvent. The IS normalised MF was calculated by dividing the MF of
196 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

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

223

224 *Dilution integrity and carryover*

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

232

233 *Intra/Inter-assay precision and accuracy*

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

242

243 *Stability*

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

254

255 *Application of method to clinical sample analysis*

256 The method was used to quantify SRA737 plasma concentrations in a patient with advanced
257 cancer treated on the ongoing Phase I clinical trial of SRA737 administered as single agent
258 monotherapy (EudraCT number 2015-004486-86). Blood samples for pharmacokinetic
259 analysis were obtained prior to administration of a single oral dose of 40 mg SRA737 and at
260 0.5, 1, 2, 4, 6, 8, 12 and 24 h post-administration. Blood samples (2 mL) were collected into
261 EDTA tubes and centrifuged at 1200 g for 5 min at 4°C. Plasma was separated and frozen at -
262 20°C prior to analysis as described above.

263

264

265 **Results & discussion**

266 *HPLC-MS/MS*

267 Using an ESI source in positive ion mode, SRA737 formed mainly a molecular ion M^+ at m/z
268 379.872, while $^{13}C^{15}N$ deuterated SRA737 (IS) formed a protonated molecule $[M+H]^+$ at m/z
269 384.086. These precursor ions passed through the first quadrupole into the collision cell and
270 the collision energy (CE) was optimized to obtain a high signal for the product ions generated.
271 After fragmentation, the characteristic products were monitored in the third quadrupole at
272 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
273 259.2 (39 eV) for the IS. The fragmentation patterns are presented in Figure 2; SRA737 and IS
274 were quantified using the transitions m/z 379.872 \rightarrow 360.200 (Figure 2A) and m/z 384.086 \rightarrow
275 324.200 (Figure 2B). Figure 3 represents typical SRM chromatograms, using the same SRA737
276 and IS transitions as above. Figure 3A shows an extracted blank plasma sample; Figure 3B
277 shows an extracted blank plasma sample with IS added; Figure 3C represents an extracted
278 plasma sample at the LLOQ (5 ng/mL) with IS. The elution of the analytes was efficient and
279 selective, SRA737 and IS being eluted at \sim 4 min. No interfering peaks were observed at these
280 retention times and the peaks were completely resolved from the plasma matrix with good
281 peak shape observed. The specificity of the method was confirmed by analysing six
282 independent sources of blank human plasma.

283

284 *Method validation*

285 *Selectivity*

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

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

290

291 *Anticoagulant comparison and matrix effect*

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

302

303 *Recovery and limit of quantification*

304 Recovery was determined in triplicate using three QC concentrations through comparison of
305 peak areas of spiked plasma samples following extraction, with peak areas obtained from
306 direct injection of SRA737 standards in mobile phase. Recovery percentages for SRA737 were
307 114%, 95.7% and 93.7% at concentrations of 30, 3000 and 15000 ng/mL, respectively, with a
308 recovery of 102% observed for the IS. There were no significant variations (1.8-8.3%) for the
309 peak areas of SRA737 and IS and all data generated were in the anticipated and acceptable
310 range. The LLOQ concentration in plasma was defined to be 5.0 ng/mL, with precision and
311 accuracy of 5.4% and 102.9%, respectively determined by preparing five plasma samples with

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

317

318 *Linearity and Range*

319 Linearity was investigated over ten concentrations of SRA737 (range 5-20000 ng/mL), with a
320 linear correlation of ≥ 0.997 calculated from 7 separate experiments. The calibration curve
321 was typically described by the linear equation $y = 1.75x + 0.003$, with $1/y^2$ weighting. The
322 weighting of $1/y^2$ gave the best linear response, with consistent % relative error values across
323 the standard curve concentration range and correlation coefficients with excellent
324 reproducibility. Table 1 shows linearity and range data over a SRA737 concentration range of
325 5-20000 ng/mL.

326

327 *Dilution integrity and carryover*

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

332

333 *Intra/Inter-assay precision, accuracy and stability*

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

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

349

350 *Clinical sample analysis*

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

358

359

360 **Conclusions & future perspective**

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

367

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

380

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

385 **Executive Summary**

386 **Background**

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

389 **Experimental**

- 390 • The method utilises small samples volumes (20µL), involving protein precipitation with
391 acetonitrile and use of ¹³C¹⁵N deuterated SRA737 as internal standard.

392 **Results and discussion**

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

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

398

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

429

Day	SRA737 plasma concentrations (ng/mL)									
	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

430

431

432

433

434

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

443

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

445

446

447

448 **Figure Legends**

449

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

452

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

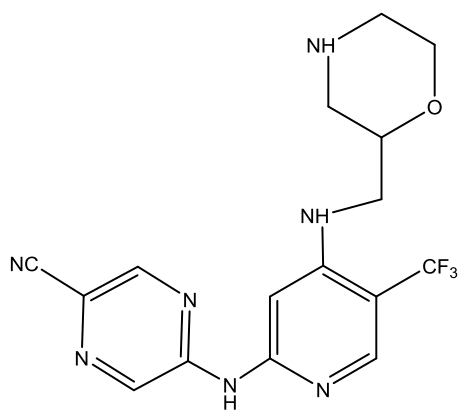
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Figure 1

465

466

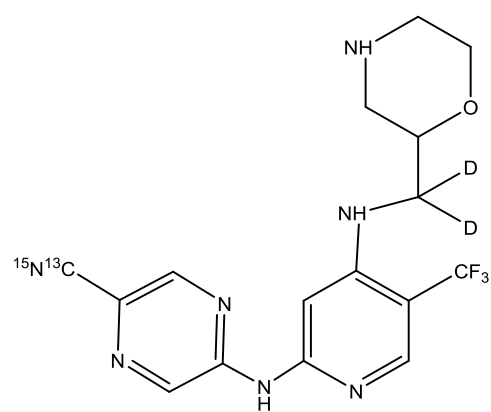
467 **A**



468

469

B



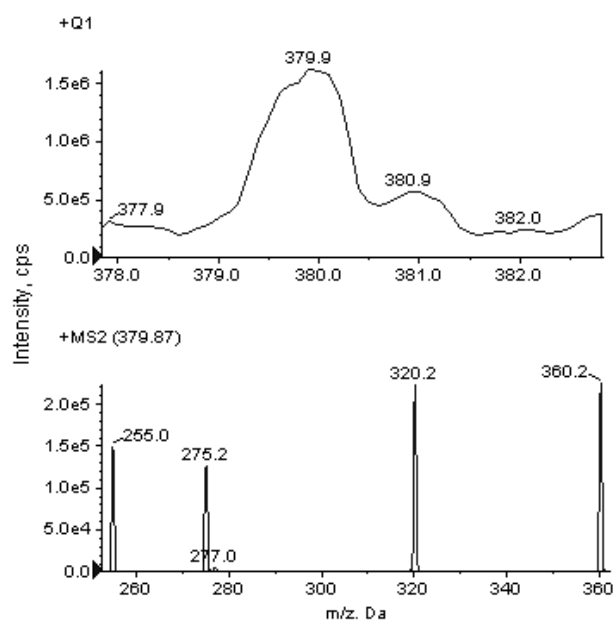
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Figure 2

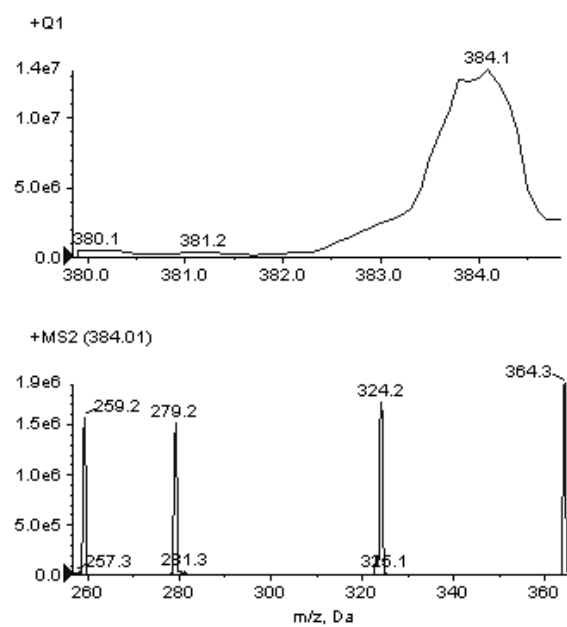
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473 **A**



B



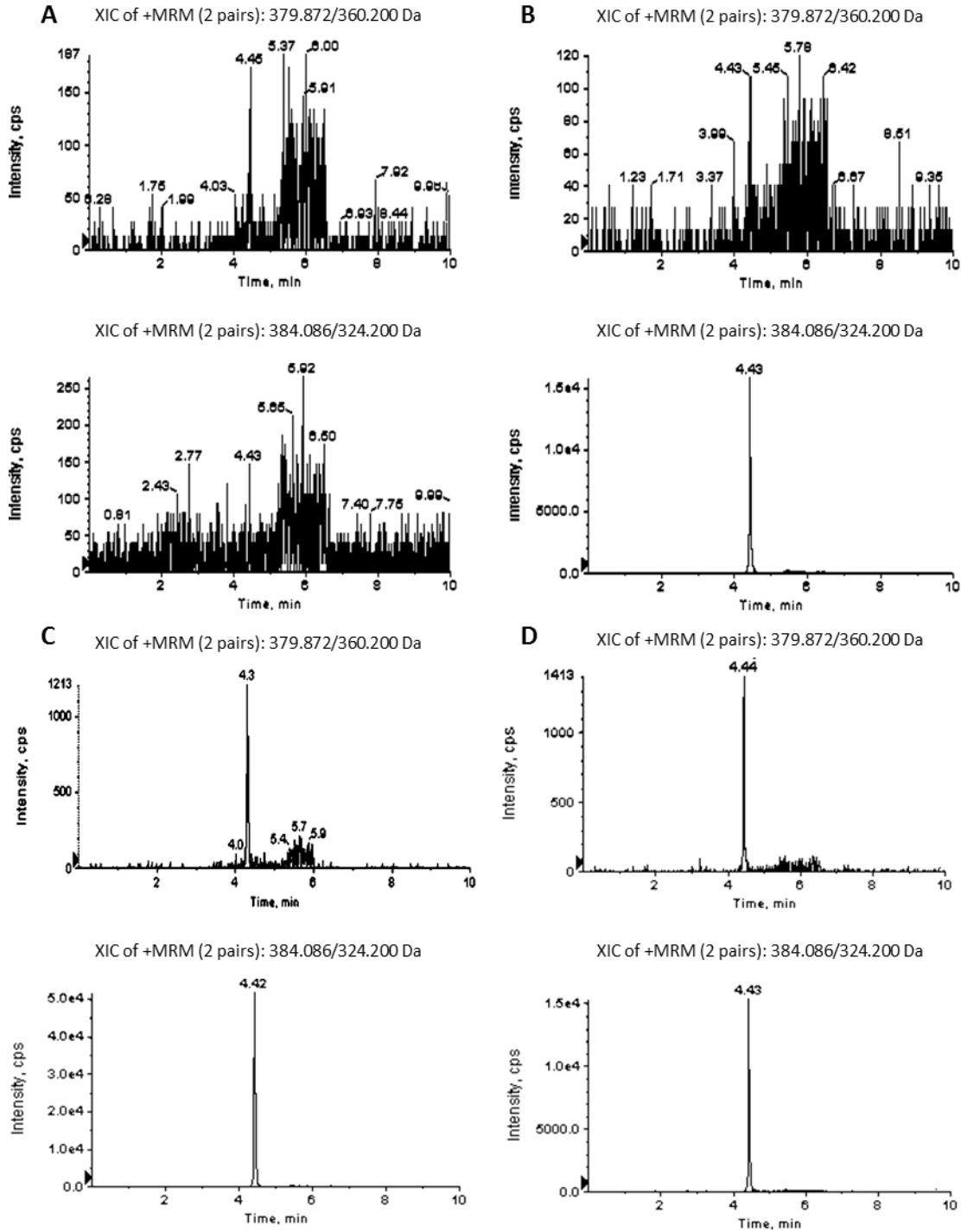
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Figure 3

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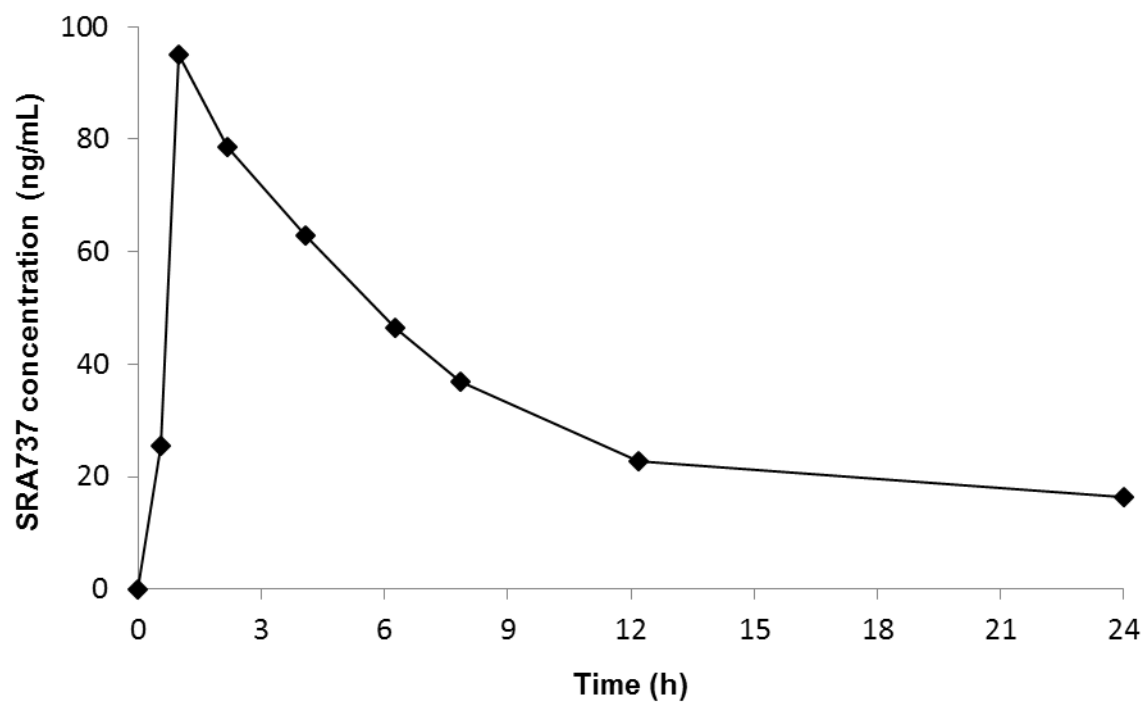
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Figure 4

482



483