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Short communication

Determination of androgen receptor degradation enhancer ASC-J9® in mouse sera and organs with liquid chromatography tandem mass spectrometry

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ABSTRACT

A novel androgen receptor (AR) degradation enhancer ASC-J9® has displayed beneficial effects during the *in vitro* and *in vivo* studies for treatment of prostate cancer, liver cancer, bladder cancer and spinal and bulbar muscular atrophy (SBMA). It works mainly *via* the degradation of AR with minimal side effects on the tested mice. Here we developed a fast, robust and more sensitive method for the quantification of ASC-J9® in 100 μL of mouse serum by using liquid chromatography tandem mass spectrometry (LC–MS/MS). The limit of quantification (LOQ) was found to be 5 nM for ASCJ9®. This method was successfully applied to investigate the pharmacokinetics of ASC-J9® in mice serum samples and also the distribution of the drug in various mice organs after single dose injection with results showing that ASC-J9® could be quickly absorbed *in vivo* and had a relatively slow elimination half-life of 5.45 h. The ASC-J9® also exhibited a higher tendency to accumulate in organs such as liver, testes and prostate.

1. Introduction

Curcumin is a major constituent of the rhizome of the turmeric herb (*Curcuma Longa Linn*) [1–3]. Chemically, it is a bis-α,β-unsaturated β-diketone and exists predominantly in keto form in the acidic or neutral conditions but a stable enol form in the alkaline condition [1,2,4]. Curcumin has been suggested as a potential anti-cancer drug through perturbation of multiple molecular targets [5] and was safe even when applied at high doses during several clinical trials [3,7,8]. However, the viability as a drug with its low oral bioavailability and its rapid *in vivo* metabolism remains as a potential problem [1,3,5–7]. One possible way of overcoming these shortcomings is to make structural modifications of curcumin [1,4–6,9], and several curcumin derivatives and analogues were developed that could also inhibit tumour cell proliferation and invasion [4,9].

In our study, we have identified a curcumin derivative, ASC-J9® (5-hydroxy-1,7-bis(3,4-dimethoxyphenyl)-1,4,6-heptatrien-3-one) as an effective androgen receptor (AR) degradation enhancer [10,11]. It can suppress AR function *via* selective interruptions between androgen receptors (ARs) and its selective co-activators (ARA55 or ARA70) that are expressed mainly in prostate stromal or epithelial luminal cells [11]. The consequences of such suppression of AR function may then lead to suppress the AR-mediated diseases, including prostate cancer (PCa) [10,11], liver cancer [12], bladder cancer [13] and spinal and bulbar muscular atrophy (SBMA) [14]. Importantly, unlike currently used androgen deprivation therapy (ADT) with various anti-androgens that may develop the castration resistant tumours, ASC-J9® can continually suppress the PCa at the castration resistant stage [10,11,15,16], with little side effects on the sexual libido of the tested mice [14]. This suggested that this drug could be a better option to treat PCa or other AR related diseases, such as liver cancer, bladder cancer or SBMA. Therefore, it is important to quickly and accurately determine the ASC-J9® concentration in various biological samples.

In this study, we have developed and validated a fast and robust LC–MS/MS method to quantify ASCJ9® in mice serum and various organ tissue samples. The pharmacokinetics and distribution of ASC-J9® in mice sera and organs have been also investigated.

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2. Experimental

2.1. Instrumentation

Chromatographic separation was carried out on an Agilent Technologies (Palo Alto, CA, USA) Model 1200 liquid chromatography system with a binary pump and an auto-sampler. Chromatographic data were recorded and processed by using the Analyst software version 1.4.2 (ABI-Sciex, Foster City, CA). A Phenomenex (Torrance, CA) Luna C18 (50 mm × 2 mm, 100 Å) column attached to a security guard column with C18 (4 mm × 2 mm) filter cartridge was used for separation. The HPLC effluent was analysed by an API 3200 triple quadrupole mass spectrometer (ABI-Sciex, Toronto, Ontario, Canada) equipped with a turbo ion spray source operated under positive multiple reaction monitoring (MRM) mode.

2.2. Chemicals

Formic acid and HPLC-grade solvents acetonitrile and ethyl acetate were purchased from Merck (Darmstadt, Germany). ASC-J9® was a kind gift from AndroScience Corporation (San Diego, CA, USA). Curcumin and bovine serum albumin (BSA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Commercial mouse serum of innovative grade was purchased from Innovative Research Inc. (Peary court Novi, MI, USA). Phosphate buffer saline (PBS) was purchased from Vivantis Technologies Sdn Bhd (Selangor Darul Ehsan, Malaysia). Ultrapure water was prepared using a Milli-Q (Millipore, Bedford, MA, USA) water purification system.

2.3. Preparation and extraction of calibration standards and quality controls

All the prepared stock and standard solutions of ASC-J9® and curcumin were stored at −20 °C and wrapped with aluminium foil. Two sets of calibration curves were established using different types of matrices including mouse sera and 1 g/L of PBS/BSA solutions. The calibration standards were prepared by spiking 20 μL of each of the standard solution of ASC-J9® in acetonitrile into either 80 μL of blank mouse serum or 80 μL of PBS/BSA solution to make up a total of 100 μL of final sample solution. As such, the final calibration standards ranged from 0.005 to 5 μM in mouse sera and 0.005 to 10 μM in 1 g/L of PBS/BSA matrix. Ten microlitres of 2.5 μM curcumin were spiked into each of the 100 μL samples as internal standards. Three quality controls with concentrations 0.025 μM, 0.25 μM and 1 μM were also prepared in both matrices. These samples were extracted using liquid–liquid extraction with 0.5 mL of ethyl acetate and vortexed vigorously for 2 min, followed by centrifuging at 13,000 rpm for 3 min at room temperature. The supernatants were transferred into clean microcentrifuge tubes. The extraction was repeated thrice and extracts were combined and dried under a gentle stream of nitrogen gas. The dried residue was reconstituted with 100 μL of 60% aqueous acetonitrile. Ten microlitres of the reconstituted solutions were injected for the analysis.

2.4. Preparation of samples for stability studies

Triplicates of the three quality controls as mentioned in Section 2.3 in both matrices were spiked with 10 μL of 2.5 μM internal standard, curcumin. These samples were stored at −80 °C and thawed at room temperature of 24 °C. They were subjected to three freeze–thaw cycles for determination of freeze–thaw stability. Another triplicate of the three quality controls were left at room temperature of 24 °C for 6, 8 and 24 h respectively before analysis to study the short term stability of ASC-J9® in mouse serum and PBS/BSA matrix under room temperature conditions. For both freeze–thaw and short term stability samples, the measurements were compared against measurements from triplicates of a reference set with the same concentrations of ASC-J9® but prepared freshly on the day of extraction and LC–MS/MS analysis.

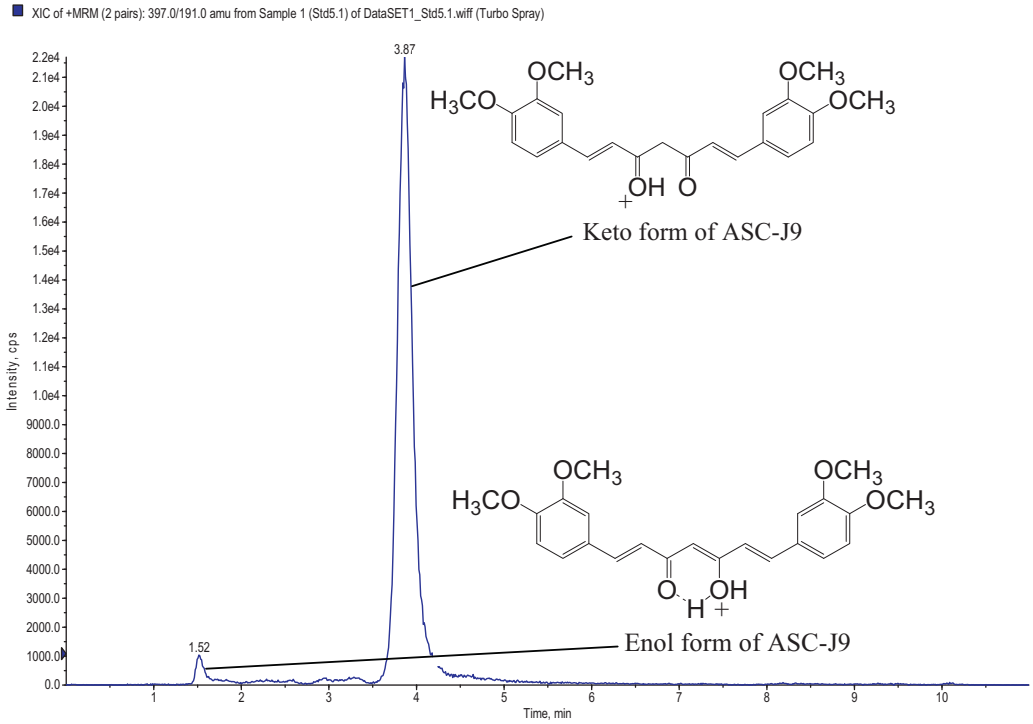


Fig. 1. Chromatogram with identification of peaks of the keto–enol isomers of dimethoxycurcumin, ASC-J9®.

Table 1a
Summary of the validation of interday calibration curves in serum matrix.

Nominal concentration (μM)	Estimated interday concentration (μM)			Interday validation summary			
	Day 1	Day 2	Day 3	Mean concentration (μM)	Stdev	CV%	Accuracy%
0.005	0.0049	0.0052	0.0050	0.0050	0.0002	3.4	100.2
0.025	0.0265	0.0244	0.0273	0.0261	0.0015	5.7	104.2
0.05	0.0508	0.0502	0.0488	0.0499	0.0010	2.1	99.8
0.25	0.2720	0.2850	0.2537	0.2701	0.0157	5.8	108.1
0.5	0.5231	0.5235	0.5013	0.5160	0.0126	2.5	103.2
1	1.0911	0.9906	1.0915	1.0577	0.0581	5.5	105.8
2.5	2.4912	2.4363	2.3523	2.4266	0.0699	2.9	97.1
5	4.2408	4.7691	5.1412	4.7170	0.4524	9.6	94.3

Summary of the validation of interday quality controls in serum matrix

Nominal concentration (μM)	Estimated interday concentration (μM)			Interday validation summary			
	Day 1	Day 2	Day 3	Mean concentration (μM)	Stdev	CV%	Accuracy%
0.025	0.0224	0.0299	0.0224	0.0249	0.0043	17.2	99.7
0.25	0.2241	0.2828	0.2566	0.2545	0.0294	11.5	101.8
1	0.9706	1.2190	0.8866	1.0254	0.1729	16.9	102.5

2.5. Sample preparation for mouse serum and organ samples

2.5.1. Mouse serum samples

All the mice were treated with 75 mg/kg of ASC-J9® through intraperitoneal (i.p.) injection. Three serum samples were taken from three different mice per timepoint at several timepoints. After which, the blood samples were left standing for 20–30 min before centrifuging at 1500 rpm for 10 min. The supernatant was then transferred to a clean tube and immediately stored at −80 °C before extraction and LC–MS/MS analysis.

2.5.2. Mouse organ samples

Triplicates of organs were collected from six mice at 8 and 24 h respectively after injection of ASC-J9®. After collection, the organs were immediately stored at −80 °C. Before extraction, the organs were thawed and rinsed with saline buffer and dried before weighing. Each organ sample was placed in a 2-ml microcentrifuge tube and homogenised. Ten microlitres of 2.5 μM curcumin were spiked as internal standard into the homogenised sample. Similar extraction procedure was adhered to as mentioned in Section 2.3 except that the tissues samples were subjected to ultrasonication for 10 min after each addition of extraction solvent. They were then centrifuged at 6000 rpm for 10 min before transferring the supernatants into clean microcentrifuge tubes.

2.6. LC–MS/MS analysis

The mobile phase consisted of two eluents, solvent A (ultrapure water with 0.1% formic acid) and solvent B (acetonitrile), delivered at a flow rate of 0.35 mL/min. The Luna C18 column was used as stationary phase for separation under isocratic mobile phase conditions of 40% solvent A and 60% solvent B. The temperature of the column compartment was kept at 40 °C. Fig. 1 is the typical chromatogram of ASC-J9® where it contains two elution peaks. This is similar to the internal standard, curcumin. These two elution peaks represented the keto–enol isomers of each of the compounds [5,9]. For both ASC-J9® and curcumin, their latter peaks were their predominant peaks (with peak intensity ratio of approximately 1:22 for ASC-J9® and 1:11 for curcumin). Since the second peaks for both ASC-J9® and curcumin were much higher than their first peaks and the ratios of the two peaks were consistent, only the second peaks for both ASC-J9® and curcumin were used for quantitation in this study.

ASC-J9® and curcumin were determined using the positive ESI-MRM mode. Both of them showed similar fragmentation and the MRM transitions for monitoring ASC-J9® was at m/z 397 → 191 and for curcumin (internal standard), it was at m/z 369 → 177. All of the source and instrument parameters were optimised by flow injection analysis. The optimised conditions for ASC-J9® were as follows: declustering potential at 30 V, entrance potential at 8 V,

Table 1b
Summary of the validation of intraday calibration curve in serum matrix.

Nominal concentration (μM)	Estimated intraday concentration (μM)						Intraday validation summary			
	1st run	2nd run	3rd run	4th run	5th run	6th run	Mean concentration (μM)	Stdev	CV%	Accuracy%
0.005	0.0062	0.0062	0.0041	0.0045	0.0053	0.0041	0.0051	0.0010	19.5	101.5
0.025	0.0313	0.0297	0.0283	0.0293	0.0290	0.0276	0.0292	0.0013	4.4	116.8
0.05	0.0540	0.0495	0.0478	0.0483	0.0425	0.0542	0.0494	0.0044	8.8	98.7
0.25	0.2360	0.2430	0.2730	0.2540	0.2470	0.2350	0.2479	0.0142	5.7	99.2
0.5	0.4960	0.4620	0.5280	0.6190	0.5820	0.5700	0.5429	0.0582	10.7	108.6
1	0.8900	1.0900	0.8120	0.9090	1.100	0.9600	0.9597	0.1143	11.9	96.0
2.5	2.9200	2.6400	2.5200	2.6600	2.6500	2.7800	2.6940	0.1395	5.2	107.8
5	4.9500	4.5400	4.1400	5.4700	4.2100	4.6300	4.6575	0.4931	10.6	93.1

Summary of the validation of intraday quality controls in serum matrix

Nominal concentration (μM)	Estimated intraday concentration (μM)						Intraday validation summary			
	1st run	2nd run	3rd run	4th run	5th run	6th run	Mean concentration (μM)	Stdev	CV%	Accuracy%
0.025	0.0294	0.0272	0.0297	0.0220	0.0225	0.0238	0.0258	0.0034	13.4	103.0
0.25	0.2540	0.2440	0.2960	0.2130	0.2280	0.2470	0.2471	0.0283	11.4	98.8
1	1.2900	1.0700	1.0600	0.9950	1.0100	0.9670	1.0644	0.1159	10.9	106.4

collisional entrance potential at 18.7 V, collisional energy at 40 V, collisional cell exit potential at 4 V, curtain gas at 10, and collisional gas at 4. The ionspray voltage was set at 5500 V with the turbo ion spray interface at 600 °C. The ion source drying gases 1 and 2 were set at 60 and 65 respectively. The concentration of ASC-J9® was quantified using an eight-point calibration curve of peak area ratio against the concentration ratio of the analyte to internal standard.

2.7. Method validation

Interday ($n = 9$) and intraday ($n = 6$) assays were performed for each set of calibration standards in the two types of matrices, mouse serum and PBS/BSA solution, to determine the accuracy and the coefficient of variation (CV%) of the experimental method. The limit of quantification (LOQ) was based on signal-to-noise (S/N) ratio of 10:1. Matrix effects were also investigated by analysing the blank mouse serum and blank PBS/BSA solution containing internal standard only.

3. Results and discussion

3.1. Validation of the LC–MS/MS method

Linear regression with $1/y^2$ was obtained for ASC-J9® in mouse serum from 0.005 to 5 μM, with regression coefficient, r^2 of 0.99 and a typical calibration curve equation of $y = 17x + 0.0584$. As for ASC-J9® in PBS/BSA matrix, quadratic regression with $1/y^2$ weighting was used to plot the calibration curves over the range of 0.005–10 μM, having the regression coefficient, r^2, of 0.99 as well and a typical calibration equation of $y = -0.58x^2 + 15.1x - 0.00286$. Recoveries and matrix effect were determined using the quality controls of ASC-J9® prepared in both mouse serum and PBS/BSA solution as matrices. From the chromatograms of both types of blank matrices, there was no peak with the same retention times as ASC-J9® at m/z 397 → 191. This shows that the method was selective and specific for detection of this curcuminoid. All the validated values for accuracies and CV% fell within acceptable requirements based on the Guidance for Industry Bioanalytical Method Validation [17] as shown in Tables 1a, 1b, 2a and 2b. The LOQ value of ASC-J9® was experimentally determined to be 0.005 μM in both matrices, having S/N ratio of at least 10:1 as calculated using the Analyst version 1.4.2 software. Compared to the reported method [9], our results indicated that our new method was more sensitive and robust and reproducible for the measurement of ASC-J9® in mouse serum and in PBS/BSA matrix and could easily be extended to other types of biological matrices.

3.2. Stability of ASC-J9® in serum and PBS/BSA matrix

It is very important to study the stability of ASC-J9® in the different matrices in order to ensure that ASC-J9® does not undergo degradation during the storage and sample preparation period. It could also give indications of metabolism for ASC-J9® when compared to curcumin. In this study, freeze–thaw and short term stability studies were conducted. For the samples prepared in serum matrix, the mean recoveries were from 90 to 105% for ASC-J9® samples with three freeze–thaw cycles when compared to the same concentrations of the freshly-spiked ASC-J9® samples (Fig. 2a). As for the samples for the short-term stability tests that were left at room temperature of 24 °C for 6, 8 and 24 h, much lower recoveries for ASC-J9® were obtained. Recoveries were 42–48% for 6 h, 30–42% for 8 h, and 13–22% for 24 h (Fig. 2b). This implied that significant degradation of ASC-J9® in serum occurred at room temperature. On the other hand, there was a relatively much lesser extent of degradation for ASC-J9® in PBS/BSA matrix than in serum for both

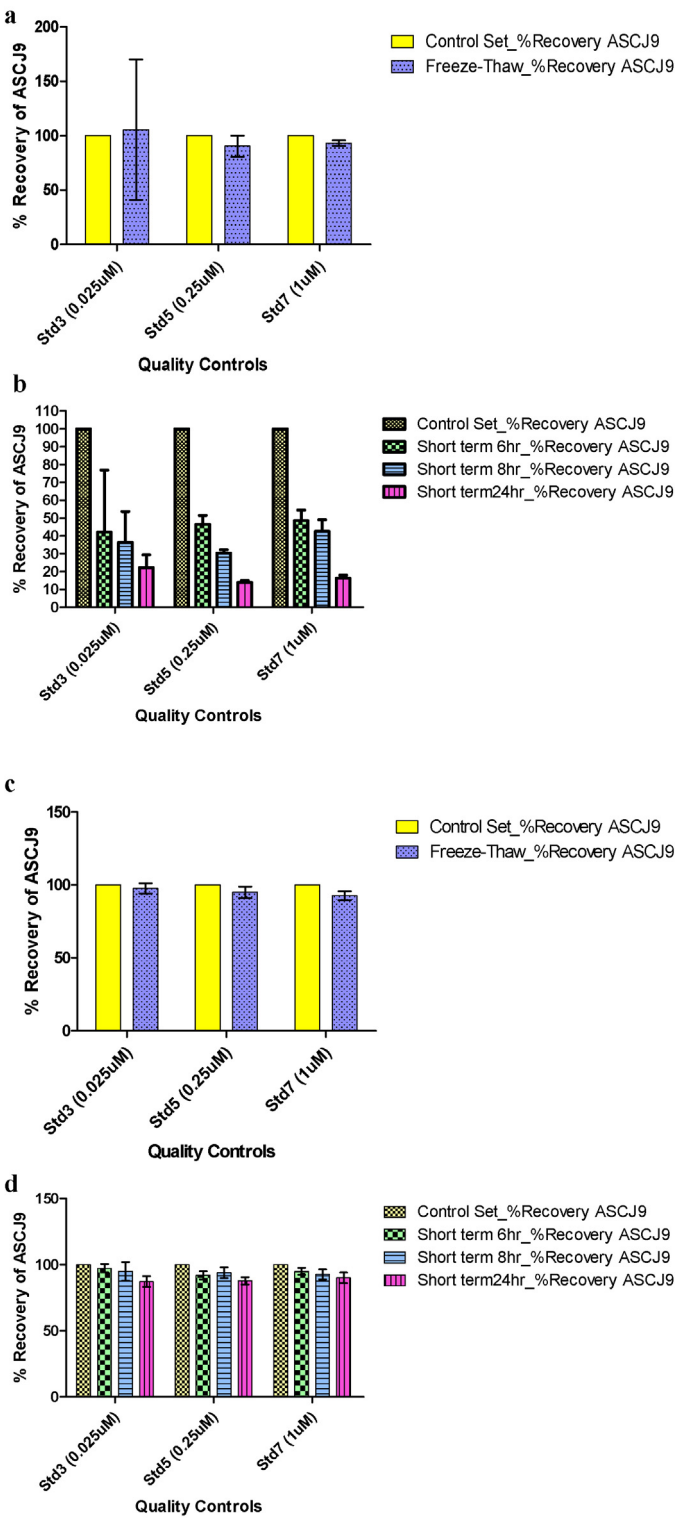


Fig. 2. Recovery data of ASC-J9® in various processing ways. (a) Recovery data of ASC-J9® in serum from freeze–thaw stability samples subjected to three freeze–thaw cycles and compared against control set freshly spiked with ASC-J9® on day of extraction. (b) Recovery data of ASC-J9® in serum from short term stability samples left at room temperature for 6, 8 and 24 h compared against control set freshly spiked with ASC-J9® on day of extraction. (c) Recovery data of ASC-J9® in 1 g/L of PBS/BSA from freeze–thaw stability samples subjected to three freeze–thaw cycles and compared against control set freshly spiked with ASC-J9® on day of extraction. (d) Recovery data of ASC-J9® in 1 g/L of PBS/BSA from short term stability samples left at room temperature for 6, 8 and 24 h compared against control set freshly spiked with ASC-J9® on day of extraction.

Table 2a
Summary of the validation of interday calibration curves in 1 g/L PBS/BSA matrix.

Nominal concentration (μM)	Estimated interday concentration (μM)			Interday validation summary			
	Day 1	Day 2	Day 3	Mean concentration (μM)	Stdev	CV%	Accuracy%
0.005	0.0049	0.0050	0.0052	0.0050	0.0001	2.2	100.4
0.025	0.0271	0.0294	0.0251	0.0272	0.0021	7.8	108.7
0.05	0.0553	0.0438	0.0449	0.0479	0.0063	13.2	96.0
0.25	0.2749	0.2318	0.2405	0.2491	0.0228	9.2	99.6
0.5	0.4939	0.5645	0.5420	0.5334	0.0361	6.8	106.7
1	0.9766	0.9309	1.1541	1.0206	0.1179	11.6	102.1
2.5	2.2628	2.6803	2.6992	2.5474	0.2467	9.7	101.9
5	4.6319	5.7530	4.9839	5.1229	0.5733	11.2	102.5
10	10.8912	9.2838	9.7128	9.9626	0.8322	8.4	99.7

Summary of the validation of interday quality controls in 1 g/L PBS/BSA matrix

Nominal concentration (μM)	Estimated interday concentration (μM)			Interday validation summary			
	Day 1	Day 2	Day 3	Mean concentration (μM)	Stdev	CV%	Accuracy%
0.025	0.0237	0.0208	0.0268	0.0238	0.0030	12.7	95.0
0.25	0.2506	0.2346	0.2768	0.2540	0.0213	8.4	101.6
1	0.9306	0.9332	0.8691	0.9110	0.0363	4.0	91.1

Table 2b
Summary of the validation of intraday calibration curve in 1 g/L PBS/BSA Matrix.

Nominal concentration (μM)	Estimated intraday concentration (μM)						Intraday validation summary			
	1st run	2nd run	3rd run	4th run	5th run	6th run	Mean concentration (μM)	Stdev	CV%	Accuracy%
0.005	0.0052	0.0054	0.0053	0.0052	0.0044	0.0055	0.0052	0.0004	7.8	103.1
0.025	0.0257	0.0255	0.0251	0.0238	0.0242	0.0236	0.0247	0.0009	3.7	98.6
0.05	0.0426	0.0428	0.0523	0.0520	0.0399	0.0399	0.0449	0.0057	12.8	89.8
0.25	0.2320	0.2290	0.2520	0.2480	0.2410	0.2350	0.2395	0.0091	3.8	95.8
0.5	0.4440	0.4420	0.6170	0.6030	0.6080	0.6060	0.5533	0.0856	15.5	110.7
1	1.1700	1.1600	1.1600	1.1700	1.1400	1.1500	1.1583	0.0117	1.0	115.8
2.5	2.7000	2.6700	2.7200	2.7600	2.7100	2.6900	2.7083	0.0306	1.1	108.3
5	4.9500	5.0500	4.9200	4.9800	5.0800	5.0700	5.0083	0.0674	1.3	100.2
10	9.5700	9.6500	9.5300	9.8600	9.7500	9.7200	9.6800	0.1220	1.3	96.8

Summary of the validation of intraday quality controls in 1 g/L PBS/BSA matrix

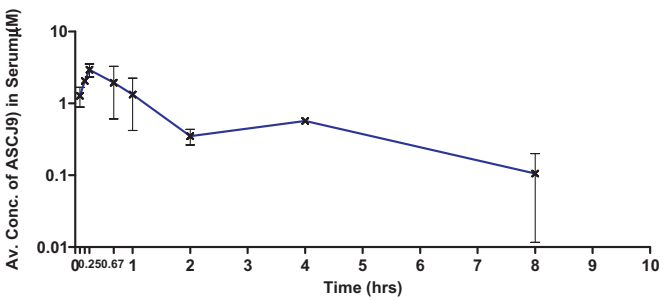
Nominal concentration (μM)	Estimated intraday concentration (μM)						Intraday validation summary			
	1st run	2nd run	3rd run	4th run	5th run	6th run	Mean concentration (μM)	Stdev	CV%	Accuracy%
0.025	0.0278	0.0246	0.0247	0.0274	0.0278	0.0250	0.0262	0.0016	6.1	104.9
0.25	0.3240	0.2290	0.2480	0.2440	0.2620	0.2510	0.2597	0.0333	12.8	103.9
1	0.8430	0.9670	0.8780	0.9120	0.8990	0.9580	0.9095	0.0473	5.2	91.0

the freeze–thaw (recoveries were 93–98% as shown in Fig. 2c) and short-term stability samples (recoveries were 92–97% for 6 h, 93–95% for 8 h, and 87–90% for 24 h as shown in Fig. 2d) conducted under the same test conditions.

When compared to ASC-J9® in serum or in PBS/BSA, curcumin degraded much faster. Under the exact conditions, curcumin was observed to undergo more significant degradation for both the freeze–thaw and short-term stability samples (data not shown). As such, to ensure that curcumin can be used as the internal standard for quantification here, it was freshly prepared and spiked into the samples just before extraction.

3.3. Pharmacokinetic study of ASC-J9® in mouse serum

This validated LC–MS/MS method was used to determine the concentration of ASC-J9® in mouse serum samples collected at different time-points for a simple pharmacokinetic study after intraperitoneal (i.p.) injection of ASC-J9® at 75 mg/kg. The pharmacokinetic estimates of ASC-J9® were calculated based on a two-compartment model using the Phoenix WinNonlin software version 6.0. The peak concentration of ASC-J9® after administration, C_{max}, was found to be 2.91 μmol/L, which occurred at 0.34 h. The concentration of ASC-J9® in serum decreased rapidly but remained detectable up to 8 h (Fig. 3). Among the other parameters obtained, the absorption half-life, $k_{01}\_t_{1/2}$, is 0.19 h, which indicated the rate at which the drug enters the systemic circulation upon intraperitoneal injection. Distribution half-life, $\alpha\_t_{1/2}$ is estimated to be 0.24 h, which indicated that ASC-J9® can be rapidly distributed in the tissues and caused the steep decrease of the concentration of



Note: Log plot was used in this case. And negligible ASC-J9 was detected at 24 hrs and thus not reflected on the plot.

Fig. 3. Pharmacokinetic diagram of ASC-J9®. (Log plot of concentration of ASC-J9® in mouse serum against time in hours) Serum samples were taken at different time points after intraperitoneal injection of 75 mg ASC-J9®/kg mouse body weight.

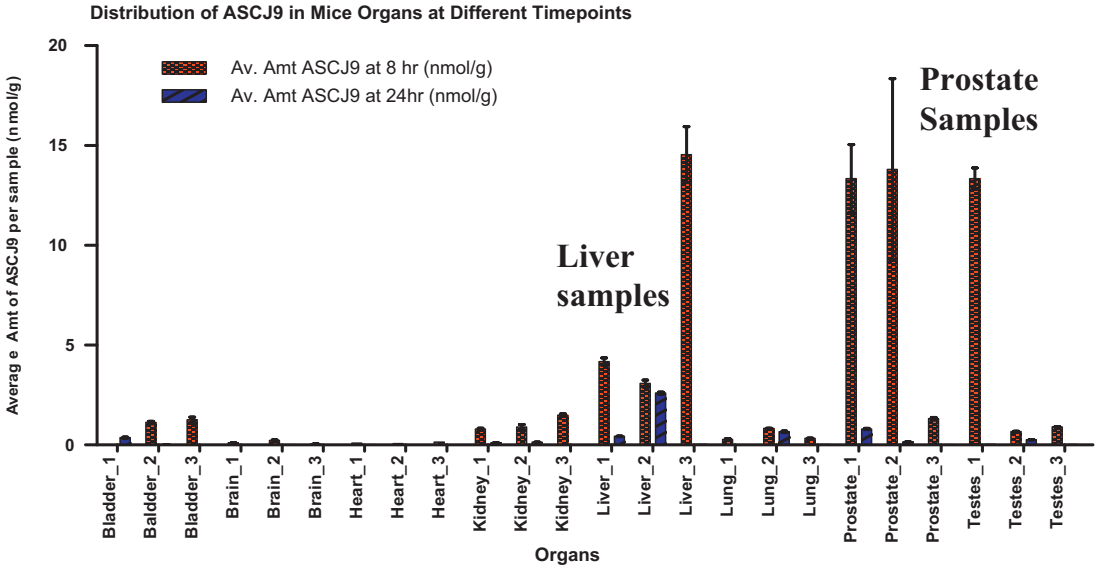


Fig. 4. Distribution of ASC-J9® in mouse organs. Mouse organs were harvested at 8 and 24 h after intra-peritoneal injection of 75 mg ASC-J9®/kg mouse body weight.

drug in serum. In addition, the elimination rate constant, β, is estimated to be 0.13 h^{-1}, which resulted in an elimination half-life, $\beta\_t_{1/2}$, of 5.45 h. This is much longer than the 0.4 h elimination half-life reported for curcumin [5]. This could imply that ASC-J9® could potentially exhibit higher bioavailability than curcumin *in vivo*, which required further confirmation.

3.4. Distribution of ASC-J9® in mouse organs at 8 and 24 h after single dose injection

At 8 h after the single injection, the concentrations of ASC-J9® remained relatively high in the prostate and the liver. This correlates to the pharmacokinetic results as discussed earlier, where the concentration of ASC-J9® in serum decreased rapidly but was quickly distributed in the organs as indicated by the short distribution half-life, $\alpha\_t_{1/2}$. Importantly, there were minimal or negligible amounts of ASC-J9® left in almost all the organs up till 24 h, as shown in Fig. 4. ASC-J9® seemed to be more likely to remain in the liver, testes and prostate with higher concentrations. However, more studies will have to be carried out to further investigate whether the accumulation of ASC-J9® in these organs after prolonged treatment could have beneficial or detrimental effects since there is no toxicology information of this drug on the organs as yet.

4. Conclusions

A simple, fast, robust and more sensitive LC–MS/MS method has been successfully developed and validated to accurately quantify the ASC-J9® concentrations in mouse serum and tissue samples. This validated method has been successfully applied to investigate the pharmacokinetics of ASC-J9® in mouse serum and the distribution of the drug in various mice organs. ASC-J9® was found to be quickly absorbed *in vivo* and had a relatively slow elimination half life of 5.45 h when compared to curcumin. The ASC-J9® has exhibited a higher tendency to accumulate in organs such as liver, testes and prostate. However, more work has to be done to explore the potential of this compound as a potent anti-cancer drug.

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