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Development and validation of a liquid chromatography–tandem mass spectrometry method for the determination of ARQ 501 (β-lapachone) in plasma and tumors from nu/nu mouse xenografts

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ABSTRACT

A sensitive and specific LC–MS/MS method employing positive electrospray ionization for the determination of ARQ 501 (β-lapachone) in (nu/nu) mouse plasma and tumor tissue is described. Samples were processed using protein precipitation with acetonitrile. A d6 analog of ARQ 501 was used as the internal standard (IS). The analytes were separated using a Zorbax SB8 column (30 mm × 2.1 mm i.d. 5 μm particle size) and analyzed in the multiple reaction monitoring (MRM) mode using mass transitions of 243 > 159 and 249 > 159 *m/z* for ARQ 501 and d6-ARQ 501, respectively. The lower limit of quantitation (LLOQ) for ARQ 501 was 3.0 ng/mL. The calibration curve was linear in the range of 3.0–2000 ng/mL with a correlation coefficient better than 0.99. Intra- and inter-batch precisions were within 8.4% for plasma and 11.8% for tumor samples. Accuracy expressed as percentage relative error (%R.E.) ranged from −9.0 to 7.7 for both plasma and tumor samples. Recovery was between 106 and 113% for both ARQ 501 and its d6 analog. Plasma pharmacokinetic data of ARQ 501 in mouse from intraperitoneal (IP) dosing at 60 mg/kg obtained using this validated method is presented along with tumor concentration data. The C_{max}, $AUC_{(0-\infty)}$, $t_{1/2}$, Cl/F, and V_d/F were determined to be 4016 ng/mL, 4392 h ng/mL, 3.9 h, 13.7 L/h/kg, and 76.5 L/kg, respectively. Tumor tissue concentrations were in the range 1–2 μM for approximately 2 h post-dose.

1. Introduction

ARQ 501 (Fig. 1) is an investigational anticancer agent that consists of a fully synthetic small molecule (3,4-dihydro-2,2-dimethyl-2*H*-naphtho[1,2-*b*]pyran-5,6-dione, β-lapachone, molecular weight 242 Da) in a stable hydroxypropyl-β-cyclodextrin (HPβCD) formulation suitable for intravenous administration. The early synthesis of β-lapachone was first described by Hooker [1–3]. However, more recently newer synthetic methodologies have been described [4]. In pre-clinical studies *in vitro* [5–8] and *in vivo* [9,10], ARQ 501 showed anti-tumor activity against a variety of human cancers. In phase Ib clinical studies, ARQ 501 showed promising anticancer activity against pancreatic cancer when administered in combination with gemcitabine. ARQ 501 is currently in several phase II cancer trials.

In cancer research, the *in vivo* pharmacology model most commonly employed is the human tumor xenograft model [11]. In this model, tumor growth is induced in the animal by explanting cultured human cancer cells or surgically excised tumor fragments from the desired cancer type into a subcutaneous pocket on the flank of an immuno-compromised animal such as the severe combined immunodeficiency (SKID) or the athymic nu/nu [12,13] mouse. HT29 cells, a human colon carcinoma cell line, have been used frequently to test drug efficacy against colon cancer [14]. Using this model, is important not only to know the concentration of a drug candidate in the plasma, but also what the concentration of the drug is at the target tissue which in this case is the tumor. In order to measure the concentrations of ARQ 501 in the plasma/tumor samples, a liquid chromatography–tandem mass spectrometry was developed. Despite the abundance of research conducted on β-lapachone, relatively few publications describe HPLC methodologies for the analysis of β-lapachone or its analogs. Steinert et al. [15] discuss an HPLC-UV reverse phase method which was used for the separation of a number naphtha[2,3-*b*]furan-4,9-diones and related compounds present in the Tabebuia avellanedae tree from which β-lapachone was originally identified. Shortly afterwards, Glen et al. [16] published an HPLC-UV reverse phase

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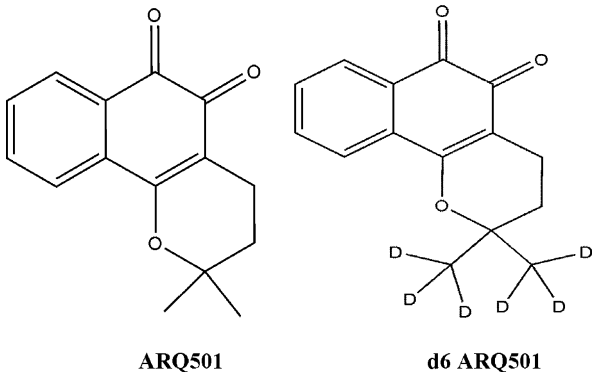

Fig. 1. Structures of ARQ 501 and d6-ARQ 501.

method for the determination of β-lapachone in human plasma samples, with a limit of detection reported as 15 ng/mL. The only other reports on an LC–MS/MS assay for β-lapachone involve its use in the analysis of aqueous solutions to determine the rates of conversion of β-lapachone prodrugs back to β-lapachone [17] and most recently, for the identification of metabolites of β-lapachone [18,19].

In order to explore the pharmaceutical properties of ARQ 501 *in vivo*, a more sensitive and specific LC–MS/MS assay was developed and validated to accurately and precisely determine the plasma concentrations of ARQ 501 in mouse samples. The method was shown to be specific, linear, accurate, and precise. Plasma pharmacokinetic data and tumor drug concentration data obtained from mice using this method are also presented.

2. Experimental

2.1. Materials

ARQ 501 and d6-ARQ 501 (Fig. 1) were prepared at ArQule Inc. in Woburn, MA. HPLC-grade water, acetonitrile, and formic acid were purchased from EMD Biosciences, in Gibbstown, NJ. Ammonium acetate was obtained from Sigma St. Louis, MO. Mouse plasma was obtained from Bioreclamation in Hicksville, NY.

2.2. Tumor homogenization and pooling

The weight of all tumor samples (obtained from nu/nu mice subcutaneously inoculated with cells from the HT-29 tumor cell line) and tumor blanks were recorded and 10 μl of 10 mM ammonium acetate was added for every milligram of tumor tissue. Tumors were homogenized using 2 mL Dounce tissue grinders (Wheaton, Millville, NJ). All blank tumors were pooled, after confirmation of absence of ARQ 501, for use in preparation of analyte standards and quality control (QC) samples. Homogenized tumors were stored at −80 °C.

2.3. Standard preparation for plasma and tumor

A 1 mg/mL stock solution of ARQ 501 was prepared in DMSO. The stock was further diluted with water to prepare a 20 μg/mL stock solution of ARQ 501 that contained 2% DMSO. Plasma or tumor homogenate standards for generation of standard calibration curves were prepared at 3, 6, 10, 30, 60, 100, 300, 600, 1000, and 2000 ng/mL by spiking the 20 μg/mL ARQ 501 stock solution into plasma or tumor homogenate.

2.4. Internal standard preparation

A 1 mg/mL stock solution of d6-ARQ 501 (100% DMSO) was diluted with acetonitrile to prepare 20 μg/mL internal standard stock solution. This stock solution was further diluted with acetonitrile to generate a 0.3 μg/mL internal standard working solution.

2.5. Tumor QC preparation

QC stock working solutions of ARQ 501 were prepared in 50/50 acetonitrile/HPLC water at 20 000 ng/mL from a 1 mg/mL stock solution of ARQ 501 in 100% DMSO. This working solution was diluted with blank tumor homogenate to generate 2000 and 1500 ng/mL QC pools. These pools were further diluted with tumor homogenate to generate QC solutions at 3, 5, and 50 ng/mL. A dilution QC at 10 000 ng/mL was prepared by spiking 1 mg/mL stock directly into blank tumor homogenate. These solutions were stored at −80 °C.

2.6. Plasma QC preparation

QC stock working solutions of ARQ 501 were prepared in 50/50 acetonitrile/HPLC water at 20,000, 2000, 500, and 50 ng/mL concentrations from a 1 mg/mL stock solution of ARQ 501 in 100% DMSO. These stocks were further diluted with nu/nu mouse plasma (anticoagulated with Na_2 EDTA) to generate QC solutions at 3, 5, 50, 500, 1500, 2000, and 10,000 ng/mL (dilution QC). These solutions were stored at −80 °C.

2.7. Plasma and tumor sample and standard preparation for LC–MS/MS analysis

For mouse plasma and tumor homogenate samples, an aliquot of 50 μL of standard, unknown, or QC was combined with 100 μL of internal standard solution (or neat acetonitrile for double blanks). Samples were centrifuged at 12,000 × *g* for 10 min and the supernatant was collected for analysis.

2.8. High performance liquid chromatography

The HPLC separation was conducted on an Agilent HP1100 HPLC system equipped with a Zorbax SB8 column (30 mm × 2.1 mm, 5 μm particle size). Mobile phase A consisted of 0.1% formic acid in water and mobile phase B consisted of 0.1% formic acid in acetonitrile. The applied gradient was as follows: mobile phase B started at 40% and was held for 0.3 min at a flow rate of 0.3 mL/min, mobile phase B was increased linearly to 95% B from 0.3 min to 2.7 min and held at 95% from 2.7 min to 3.25 min, finally, the gradient was set back to 40% B and the system was allowed to equilibrate. The column and autosampler were at ambient temperature. The injection volume was 10 μL. The retention times of ARQ 501 and d6-ARQ 501 were both approximately 1.8 ± 0.05 min. The total run time was 3.75 min. The HPLC flow was diverted to waste prior to 0.3 min and after 2.7 min to minimize source fouling.

2.9. Tandem mass spectrometry

Mass spectrometry detection was executed on a Waters Micromass Ultima LC triple quadrupole mass spectrometer equipped with an electrospray probe. Analyses were conducted in the positive electrospray multiple reaction monitoring (MRM) mode with a desolvation temperature of 350 °C and a capillary voltage of 3500 V. The source temperature was 110 °C and the cone voltage was 35 V. The nebulizer and drying gas flows were ∼70 and ∼700 l/h, respectively. The collision cell voltage was set to 20 V and the collision cell

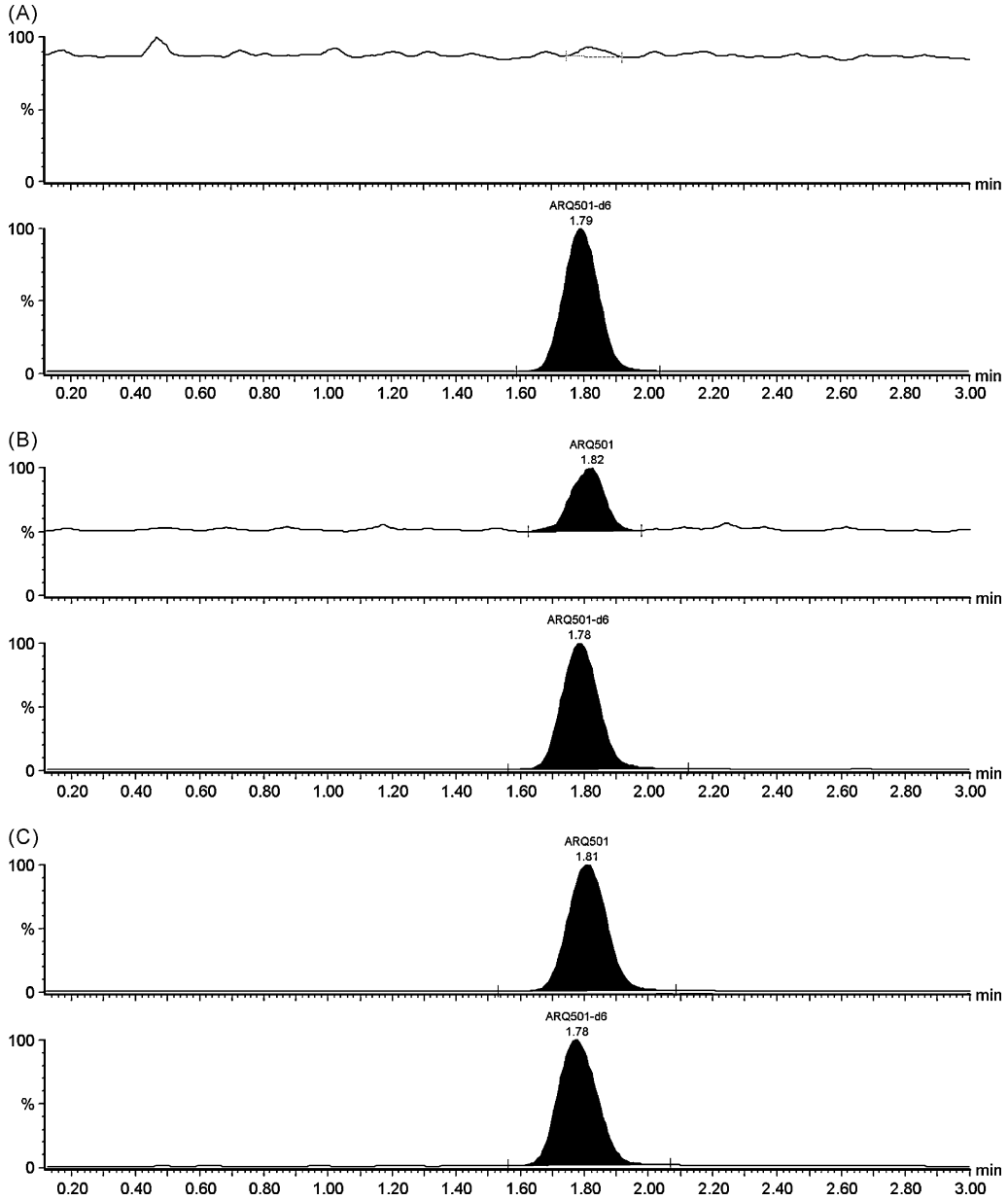

Fig. 2. Chromatogram of a: (A) blank containing only d6-ARQ 501 (B) 3 ng/mL standard with internal standard and (C) 2000 ng/mL standard with internal standard.

gas pressure was set to $\sim 3.5 \times 10^{-3}$ Torr by introducing argon into the collision cell. The MRM transitions monitored were 243 > 159 and 249 > 159 m/z for ARQ 501 and d6-ARQ 501, respectively.

2.10. Method validation

The procedures and criteria used for validation of the method were based on the FDA guidance document on bioanalytical method validation [20]. The specificity of the method was evaluated by analyzing blank plasma from six different lots of mouse plasma and examining the chromatogram region corresponding to the retention time of the analyte and IS for interferences. For tumor sample specificity, tumors from at least six different mice were evaluated. Additionally, specificity was further evaluated by preparing six separate QC samples at the lower limit of quantification (LLOQ) and assuring that the coefficient of variation (CV) was within +/−20%. Recovery was evaluated by comparing analyte (or IS) peak areas from extracted QC samples to those of spiking solutions prepared at the same concentrations. The accuracy and precision of the method was evaluated for both QC and standard samples from six replicates and multiple batch analyses of both QC and standards over the linear concentration range of the method. Intra-batch accuracy and precision was determined on a single day from six independent preparations of QCs and standards. Additional inter-batch precision and accuracy was determined by performing the intra-batch accuracy on three separate days and calculating the accuracy and precision for all 18 of the standard and QC samples at each concentration. The criteria for accuracy (%R.E.) and precision are within +/−20% at the LLOQ and within +/−15% at all other concentrations. Stability was evaluated using QC samples after storage at −80 °C for 50 days, after three-freeze–thaw cycles, and for processed samples after 24 h in the autosampler by running the QC samples along with freshly prepared standards.

Table 1
QC sample intra-assay precision and accuracy for the quantitation of ARQ 501 in mouse plasma and tumor homogenate ($n = 6$)

	QC levels						
	LLOQ	LOW1	LOW2	MED1	HIGH	HIGH	Dilution
	3[a]	5[a]	50[a]	500[a]	1500[a]	2000[a]	10,000[a]
Plasma							
Mean (ng/mL)	2.9	4.6	51.2	491.4	1559.6	1951.3	10,663
Precision (%R.S.D.)	6.3	8.4	5.6	0.9	1.3	2.5	4.1
Accuracy (%R.E.)	−2.2	−9.0	2.5	−1.7	4	−2.4	6.6
Tumor homogenate							
Mean (ng/mL)	3.0	5.0	49.4	X	1608.4	2153.7	9890.5
Precision (%R.S.D.)	11.8	4.8	2.5	X	3.4	2.0	4.7
Accuracy (%R.E.)	−1.1	−0.3	−1.2	X	7.2	7.7	1.1

[a] Concentration (ng/mL).

2.11. In vivo pharmacokinetic studies in nu/nu mice

Pharmacokinetic studies were conducted on 8-week-old female athymic (Ncr nu/nu) mice ($n = 24$) from Charles River Laboratories (Wilmington, MA) which were approximately 25 g in weight. The rats were inoculated subcutaneously with $\sim 5 \times 10^6$ cells from HT29 human colon cancer cells and the tumors were allowed to grow (~1 week) until reaching ~200–300 mm^3 in size. Animals were fasted overnight (~12 h) and dosed the following morning intraperitoneally with 60 mg/kg ARQ 501 formulated 40% HPβCD. All 24 mice were dosed and triplicate ($n = 3$ mice) blood samples were collected via cardiac puncture at predose, 0.25, 1, 2, 4, 8, 12, and 25 h into tubes containing Na_2 EDTA. Plasma was collected from the blood samples by centrifugation at $1500 \times g$ for 10 min and stored at −80 °C until analysis. Tumor samples were removed from the mice, flash frozen in liquid N_2, and stored at −80 °C until analysis. Noncompartmental pharmacokinetic parameters were calculated from plasma concentration–time data using WinNonlin (Pharsight, Mountain View, CA).

3. Results

3.1. Method development

The positive electrospray ionization mode was used for analyses because ARQ 501 does not ionize in the negative electrospray mode. Infusion experiments with ARQ 501 showed that infusion solutions containing formic acid improved the signal response of ARQ 501, therefore 0.1% formic acid was added to the mobile phase. The ARQ 501 product ion spectrum showed that the 159.1 m/z transition was the most abundant and therefore the 243.1 > 159.1 m/z mass transition was selected for quantitation. Similarly, based on the product ion spectrum, the 249.1 > 159.1 m/z mass transition was selected as the quantitation ion for d6-ARQ 501.

Table 2
QC sample inter-assay precision and accuracy for the quantitation of ARQ 501 in mouse plasma ($n = 18$) and tumor homogenate

	QC levels			
	LOW1	LOW2	MED	HIGH
	5[a]	50[a]	500[a]	1500[a]
Plasma				
Mean (ng/mL)	4.7	52.2	516.8	1601.7
Precision (%R.S.D.)	8.1	7.2	5.1	3.9
Accuracy (%R.E.)	−6.3	4.4	3.4	6.8
Tumor				
Mean (ng/mL)	5.1	50.1	X	1575.1
Precision (%R.S.D.)	6.2	3.2	X	3.9
Accuracy (%R.E.)	1.0	0.1	X	5.0
n	12	18	X	18

Note: X = not performed.
[a] Concentration (ng/mL).

3.2. Method validation

The LLOQ in mouse plasma and tumor homogenate for ARQ 501 was 3 ng/mL. The assay was linear in the range of 3–2000 ng/mL with a correlation coefficient (r) greater than 0.99. Sample chromatograms are shown for an IS blank, and both 3 and 2000 ng/mL standards in Fig. 2. The method was validated for quantitation of samples up to 10 000 ng/mL by preparing and qualifying a dilution QC at 10 000 ng/mL.

Plasma intra-assay precision values ($n = 6$), based upon relative standard deviation of QC samples prepared at 3, 5, 50, 500, 1500, 2000, and 10,000 ng/mL were less than or equal to 8.4%. Intra-assay accuracy values, based upon relative error ranged from −9.0 to 6.6%. These data and individual mean data are summarized in Table 1. Tumor intra-assay precision values ($n = 6$), based upon relative standard deviation of QC samples prepared at 3, 5, 50, 1500, 2000, and 10,000 ng/mL were less than or equal to 11.8%. Intra-assay accuracy

Table 3
Standard inter-assay precision and accuracy for the quantitation of ARQ 501 in mouse plasma ($n = 4$) and tumor homogenate ($n = 7$)

	Concentration (ng/mL)									
	3	6	10	30	60	100	300	600	1000	2000
Plasma										
Mean (ng/mL)	2.9	6.1	10.6	30.9	61.3	103.7	302.8	582.3	962.6	1890.0
Precision (%R.S.D.)	5.2	6.2	0.7	3.2	2.5	3.1	0.9	1.3	1.6	0.8
Accuracy (%R.E.)	−4.2	2.1	5.6	2.8	2.2	3.7	0.9	−2.9	−3.7	−5.5
Tumor homogenate										
Mean (ng/mL)	3.0	6.1	9.8	30.0	61.6	102.2	298.5	582.0	989.4	1993.3
Precision (%R.S.D.)	4.8	4.6	3.8	8.5	3.5	3.3	5.0	4.1	6.9	7.1
Accuracy (%R.E.)	0.2	−1.8	1.8	0.1	−2.7	−2.2	0.5	3.0	1.1	0.3

Table 4
Pharmacokinetic parameters of ARQ 501 in mouse

Pharmacokinetic parameter	IP administration
C_{max} (ng/mL)	4016
$t_{1/2}$ (h)	3.9
$AUC_{(0-\infty)}$ (h ng/mL)	4392
Cl/F (L/h/kg)	13.7
V_d/F (L/kg)	76.5

values, based upon relative error ranged from −1.2 to 7.7%. These data and individual mean data are summarized in Table 1. Plasma inter-assay precision values (n = 18), based upon relative standard deviation of QC samples prepared at 5, 50, 500, and 1500 ng/mL were less than or equal to 8.1%. Inter-assay accuracy values, based upon relative error ranged from −6.3 to 6.8%. These data and individual mean data are summarized in Table 2. Plasma inter-assay precision values (n = 4), based upon relative standard deviation of calibration standard standards prepared at 3, 6, 10, 30, 60, 100, 300, 600, 1000, and 2000 ng/mL were less than or equal to 6.2%. Plasma inter-assay accuracy values, based upon relative error ranged from −5.5 to 5.6%. These data and individual mean data are summarized in Table 3. Tumor inter-assay precision values (QC1 n = 12, QC 2 and 3 n = 18), based upon relative standard deviation of QC samples prepared at 5, 50, and 1500 ng/mL were less than or equal to 6.2%. Tumor inter-assay accuracy values, based upon relative error ranged from 0.1 to 5.0%. These data and individual mean data are summarized in Table 2. Tumor inter-assay precision values (n = 7), based upon relative standard deviation of calibration standard prepared at 3, 6, 10, 30, 60, 100, 300, 600, 1000, and 2000 ng/mL were less than or equal to 8.5%. Tumor inter-assay accuracy values, based upon relative error ranged from −2.7 to 3.0%. These data and individual mean data are summarized in Table 3.

The specificity of this analytical method was determined by analyzing six different lots of nu/nu mouse plasma from Bioreclamation, Inc. No interference to ARQ 501 was observed in any of the lots. Additionally, the six lots of nu/nu mouse plasma were each spiked at 200 ng/mL with ARQ 501 and analyzed. No significant difference in response was observed for any of the lots.

The recovery of ARQ 501 was determined to be slightly greater than 100% (113% recovery at 5 ng/mL and 106% ng/mL recovery at 500 ng/mL, respectively). This may have been the result of a slight matrix effect as demonstrated by the observation of a slight signal enhancement (approximately 7%). However, as shown by the accuracy, precision, and linearity of the method and the fact that d6-ARQ 501 is used as the internal standard, this apparent matrix effect has no impact on the quantitation of ARQ 501 in mouse plasma.

Plasma samples containing ARQ 501 have been shown to remain stable at room temperature for at least 24 h and can undergo at least three-freeze–thaw cycles. Long term stability of ARQ 501 in mouse plasma was confirmed for at least 50 days at −80 °C. Extracted samples are stable for at least 24 h at room temperature.

3.3. Pharmacokinetic data

The method described above was successfully used to determine the plasma pharmacokinetics of ARQ 501 in mice and to determine respective tumor concentrations. Fig. 3 shows the plasma concentration versus time profile from mice dosed with 60 mg/kg intraperitoneal (IP) ARQ 501. Based on this profile, the pharmacokinetic parameters were calculated using Winnonlin and are summarized in Table 4. Fig. 4 shows a comparison of the concentration time profiles of ARQ 501 in plasma and in tumor.

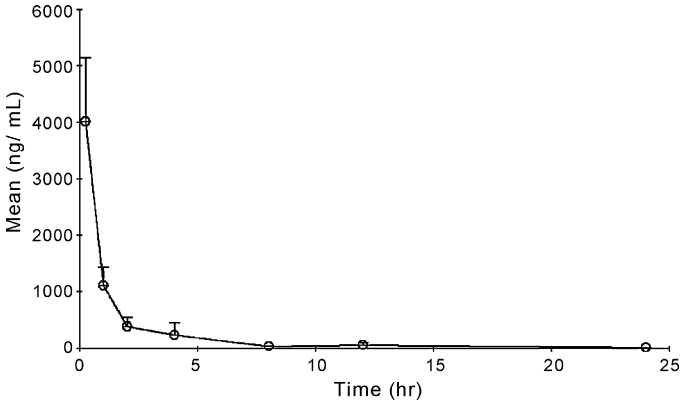

Fig. 3. Plasma concentration versus time profile for mice dosed with 60 mg/kg IP ARQ 501.

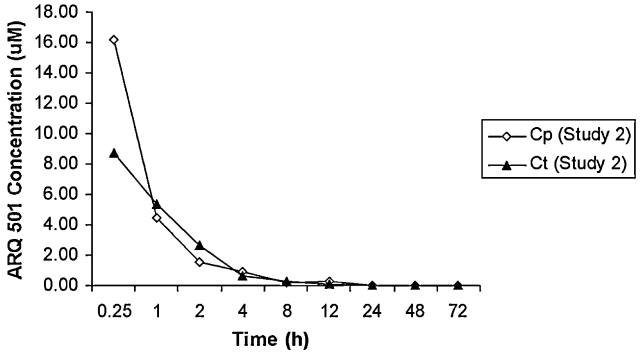

Fig. 4. Comparison of concentration time profiles for ARQ 501 in mouse plasma and mouse tumor.

4. Conclusion

An LC–MS/MS assay of ARQ 501 in biological matrices including plasma and tumor tissues is presented in this report. The assay was established and confirmed to be sensitive, linear, accurate, precise, and specific and was successfully employed in mouse xenograft studies to determine concentrations of ARQ 501 in plasma and in tumor tissue. The data reported here demonstrated that ARQ 501 was reaching adequate plasma and tumor concentrations for efficacy to be achieved [8].

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