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Simultaneous determination of doxifluridine and 5-fluorouracil in monkey serum by high performance liquid chromatography with tandem mass spectrometry

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ABSTRACT

A reverse-phase high performance liquid chromatography method with electrospray ionization and detection by mass spectrometry is described for the simultaneous determination of doxifluridine and its active metabolite 5-fluorouracil in monkey serum. A liquid/liquid extraction with ethyl acetate (90%) and isopropyl alcohol (10%) was used to extract simultaneously doxifluridine and 5-FU which have considerable difference in the polarity. Optimum chromatographic separation was achieved on a Agilent Zorbax C_{18} (100 mm × 2.1 mm, 3.5 μm) column with a mobile phase of methanol–water (20:80, v/v). The flow rate was 0.2 mL/min with total cycle time of 5 min. The lower limit of quantification (LLOQ) was validated at 10.0 ng/mL of serum for both doxifluridine and 5-FU. Accuracy and precision of quality control (QC) samples for both compounds met FDA Guidance criteria of ±15% with average QC accuracy of 95.5–105.0% and coefficients of variation of 1.1–9.5% in the 10–2000 ng/mL concentration range. This method demonstrated adequate sensitivity, specificity, accuracy, precision, stability to support the analysis of monkey serum samples.

1. Introduction

Doxifluridine, 5-deoxy-5-fluorouridine, is an orally administered prodrug of 5-fluorouracil (5-FU) which is converted into 5-FU in the presence of thymidine phosphorylase (TP) [1]. 5-Fluorouracil (5-FU), a pyrimidine analog, has been widely used in the treatment of various solid tumors for more than 20 years, and is still one of the most commonly used in the chemotherapeutic treatment of patients with breast and colon cancer. 5-FU's inhibition of thymidylate synthase and incorporation into nucleic acids are responsible for the cytotoxic activity [2]. Doxifluridine has been developed to improve therapeutic index of 5-FU and to reduce the toxicity of 5-FU. It has been reported in animal models [3] and in patients [4] that metabolism of doxifluridine to 5-FU is preferential in tumors because the concentration of TP is higher in tumor tissue compared to normal tissue.

Several HPLC methods have been developed to study only 5-FU in plasma or tissues [5–7]. The difference in polarity between doxifluridine and 5-FU has prevented a same extraction or the simultaneous analysis. A HPLC method with UV detection for simultaneous determination of doxifluridine and 5-FU has been reported, which requires different extraction procedures for the two compounds and a long LC run time more than 20 min a sample [8,9]. Although a LC–tandem MS spectrometry was used for simultaneous determination of doxifluridine and 5-FU, a LC analysis cycle time was still greater than 10 min with a LLOQ of 50 ng/mL for 5-FU [10]. Their method is not sufficient for the determination of 5-FU, as an active metabolite of doxifluridine. Since the metabolism ratio of doxifluridine into 5-FU was approximately 10^{-1} in animal study, the blood 5-FU concentration is much lower than the parent drug, doxifluridine [11].

The present paper reports the development and validation of an electrospray LC–MS/MS method for the simultaneous determination of doxifluridine and 5-FU in monkey serum. An effective liquid–liquid extraction was developed to extract both doxifluridine and 5-FU in a procedure. An optimistic organic solvent combination was investigated to extract doxifluridine and 5-FU simultaneously from the blood matrix, because doxifluridine is very soluble in water and the polarity of doxifluridine and 5-FU is quite a different; a very simple extraction procedure with the selection of ethyl acetate and *iso*-propyl alcohol in 0.05 M HCl acidic condition was successfully developed. Chromatographic separation was achieved on a reverse-phase column with an aqueous mobile phase.

This study addresses selectivity, linearity, accuracy, precision, over-range sample analysis, recovery, and analyte stability for validation of the developed analytical method for simultaneous determination of doxifluridine and 5-FU in monkey serum and also

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reports the application of this method to toxicokinetic study of doxifluridine in monkey.

2. Experimental

2.1. Materials

Doxifluridine (purity, 99.3%) was provided by Shinpoong Pharm. (Seoul, Korea). 5-FU and 5-chlorouracil (internal standard) were obtained from Sigma (MA, USA). The HPLC grade methanol was purchased from J.T. Baker (NJ, USA). Hydrochloric acid was Sigma product (MA, USA). All aqueous solutions were prepared in Mili-Q water (Millipore, MA, USA).

2.2. Standards and quality control (QC) samples

The standard stock solutions of 100 μg/mL of doxifluridine and 5-FU were prepared in 20% methanol in water (mobile phase) and 100% methanol, respectively. A series of standard working solutions were obtained by further dilution of the standard stock solutions with 20% methanol. Internal standard working solution (1000 ng/mL) was prepared by diluting internal standard stock solution (100 μg/mL) with 100% methanol.

The calibration standards were freshly prepared by spiking an appropriate amount of the standard working solution into monkey serum, at the ratio of 1/10. Eight non-zero calibration standards were prepared at concentrations of 10, 20, 50, 100, 200, 500, 1000, and 2000 ng/mL. QC samples were prepared at concentrations of 30, 400, and 1600 ng/mL.

2.3. Sample preparation

Fifty microliters of serum calibration standards, serum blank, and QC samples was added to 1 mL of 0.05 M HCl solution with 50 μL of internal standard (5-CU) spiking solution. To extract doxifluridine, 5-FU and internal standard (5-CU) effectively which are different in the polarity, 5 mL of ethyl acetate/*iso*-propyl alcohol (9/1) was added and mixed using a vortex mixer for 10 min. After centrifugation at 3000 rpm for 5 min at 4 °C, 4 mL of the supernatant was transferred and evaporated to dryness using a centrifuge vacuum system at 60 °C. The dried extract was resuspended in 250 μL of mobile phase and 10 μL were analyzed by HPLC.

2.4. Chromatographic condition

An Agilent HP 1100 series liquid chromatography consisting of a solvent degasser, binary pumps, an auto-sampler, a column heater, and an Agilent Zorbax C_{18} (100 mm × 2.1 mm, 3.5 μm particle size) column with Agilent Zorbax C_8 (18 mm × 2.1 mm, 3.5 μm particle size) guard column were used for the chromatographic separation of doxifluridine, 5-FU, and an internal standard, 5-chlorouracil (5-CU). The mobile phase was Methanol-Water (20:80, v/v) at a flow rate of 0.2 mL/min. The method used isocratic elution with a total run time of 5 min. The column and auto-sampler tray temperature were maintained at 40 °C and 4 °C, respectively.

2.5. Mass spectrometric condition

An API 3200 Q trap mass spectrometer (Applied Biosystems, USA) with an electrospray ionization (ESI) interface operated in negative mode was used for the multiple reaction monitoring (MRM) LC–MS/MS analysis. The optimized instrument conditions were as follows: source temperature, 500 °C; curtain gas, 20 psi; nebulizing (GS1), 50 psi; heating (GS2), 50 psi; collision energy (CE), −28 V for doxifluridine, −30 V for 5-FU, and −28 V for 5-CU, respectively. The following precursor and product ion transitions were used for multiple reaction monitoring: doxifluridine, 245 → 108: 5-FU, 129 → 42: 5-CU, 145 → 42. The dwell time for each transition was 250 ms. Peak-area ratios obtained from the MRM chromatograms of the analyte compound (m/z 245 → 108 for doxifluridine and m/z 129 → 42 for 5-FU) and the internal standard (145 → 42) were used for quantitation. Both quadrupoles were maintained at unit mass resolution. Analyst software (Ver. 1.4) was used for instrumentation control and data collection.

2.6. Method validation

The selectivity of method was evaluated by six lots of blank serum. The reproducibility of these six blank matrices, zero samples and LLOQ samples was used to evaluate the presence or absence of interference. Calibration curves containing standards at concentrations of 10, 20, 50, 100, 200, 500, 1000, and 2000 ng/mL were constructed by plotting the peak-area ratios of analyte/IS to the nominal concentrations. Calibration curves were analyzed by weighted linear regression analysis ($1/x$). Three validation batches were used to assess the precision and accuracy of the method. Each batch was processed on a separate day and has one set of calibration and six replicates of QC samples at 30, 400, and 1600 ng/mL. Recovery was determined by comparing the LC–MS/MS response of extracted normal QC samples at low (30 ng/mL), middle (400 ng/mL), and high (1600 ng/mL) concentration levels with that obtained from the extracted blank samples spiked with the standard solutions at the same concentration [12,13]. Stabilities of stock solution, freeze–thaw cycles, and post-preparative samples were evaluated. The concentration–time profiling of doxifluridine in addition to typical short-term stability were performed because doxifluridine decreases after spiked into serum.

2.7. Animal study

Three male cygnomolgus monkeys (*Macaca fascicularis*) aged 4–6 years old were used for toxicokinetic data. The animal room was maintained at a temperature of 23 ± 3 °C, a relative humidity of 55 ± 10%, approximately 12 h light/dark cycle with 150–300 lx, and ventilation 10–20 times/h. A standard primate diet (Oriental Yeast, Co. Ltd., Japan) was provided and the drinking water was filtered and irradiated by ultraviolet light. These animals were fasted for 16 h prior to dosing and given free access to water.

A dosing solution (20 mg/mL) was formulated in water since doxifluridine is very soluble in water. Monkeys were dosed orally at 100 mg/kg of doxifluridine via an oral catheter. Blood samples were taken from vein blood at each scheduled time (pre-dose (0), 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8 and 10 h) after dosing. Blood serum samples were obtained after centrifugation. The samples were analyzed by the developed LC/MS/MS method. All animal experimental procedures were performed in an AAALAC accredited facility.

3. Results and discussion

3.1. Mass spectra and chromatography

The chemical structures of doxifluridine, 5-FU and an internal standard, 5-CU were displayed in Fig. 1 along with the corresponding product ion scan mass spectra.

Several dominant ions become candidate ions for a MRM condition, the intensity of product ion is changed to vary the collision energy. For doxiflurdine, the 245/128 and 245/108 ion-pairs were investigated to find a MRM condition that give better quantitative and selective results. Though the 128 ion was dominant for product

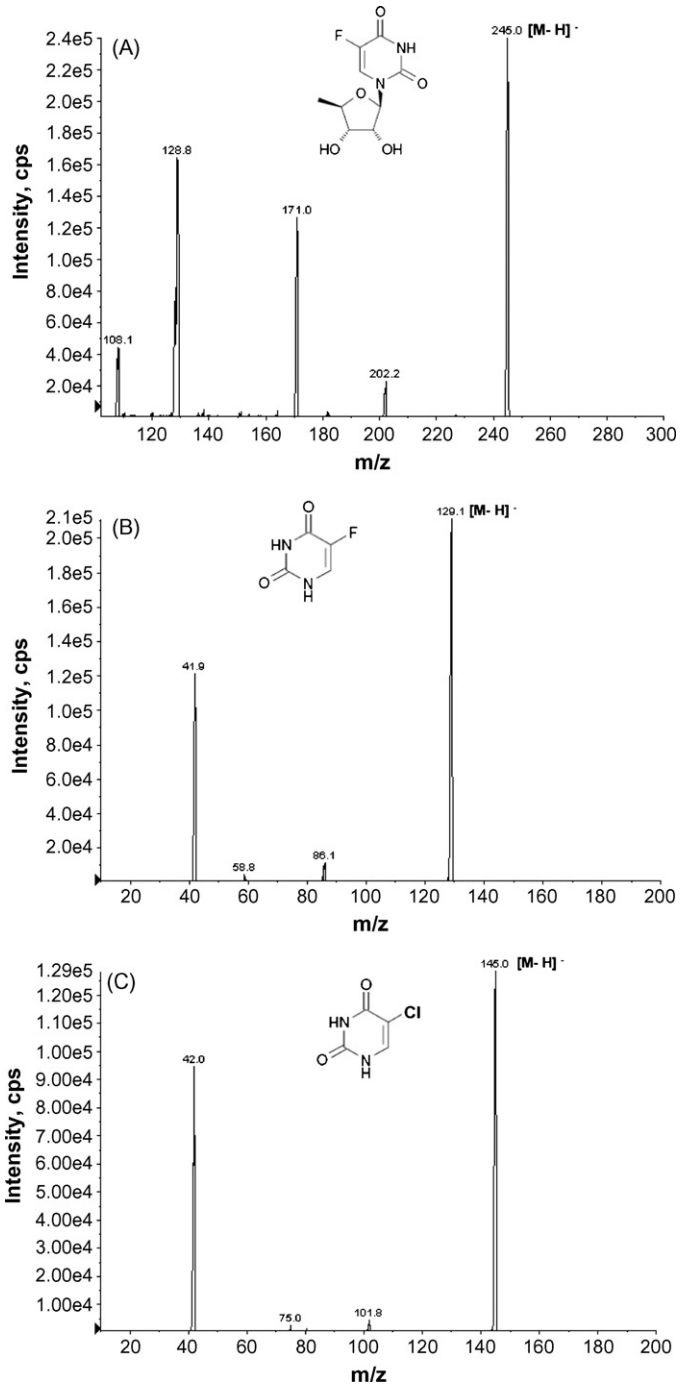

Fig. 1. Chemical structures and product ion scan MS spectrum of (A) doxifluridine, (B) 5-FU and (C) 5-CU.

ion scan, the 245/108 ion pairs condition has better accuracy and selectivity.

The transition m/z 129 → 42 were employed for the detection and quantitation of 5-FU, respectively. The internal standard, 5-CU was monitored using the transition m/z 145 → 42. For analysis in MRM mode, the transitions were optimized by the adjustments of collision energy, declustering potential, and entrance potential.

For the simultaneous determination of doxifluridine, 5-FU and internal standard, chromatographic conditions were developed to add selectivity to the validation and also to elute all three analytes with good shapes in a short running time. All three compounds eluted between 1 and 3 min after injection. The injection-to-injection cycle time was 5 min. The retention times for doxifluridine, 5-FU and 5-CU were 2.8, 1.6, and 1.9 min, respectively. Typical peak shapes and retention times of the MRM chromatograms are shown in Fig. 2.

3.2. Selectivity and lower limit of quantification

Doxifluridine and 5-FU were clearly separated from interferences in the blank matrix under the current LC–MS/MS conditions. The chromatograms of six lots of blank serum were found to contain no endogenous peak co-eluted with any of analytes and internal standards.

The lower limits of quantification for both doxifluridine and 5-FU in monkey serum was 10 ng/mL. The accuracy and precision of five replicates for doxifluridine were 97.6 and 6.4%, respectively and those for 5-FU were 104.8 and 9.5%, respectively, as listed in Table 1.

3.3. Linearity

The method was linear over the concentration range 10–2000 ng/mL in serum. Linear coefficient of correlation was higher than 0.999 for three batches. Table 2 shows the data

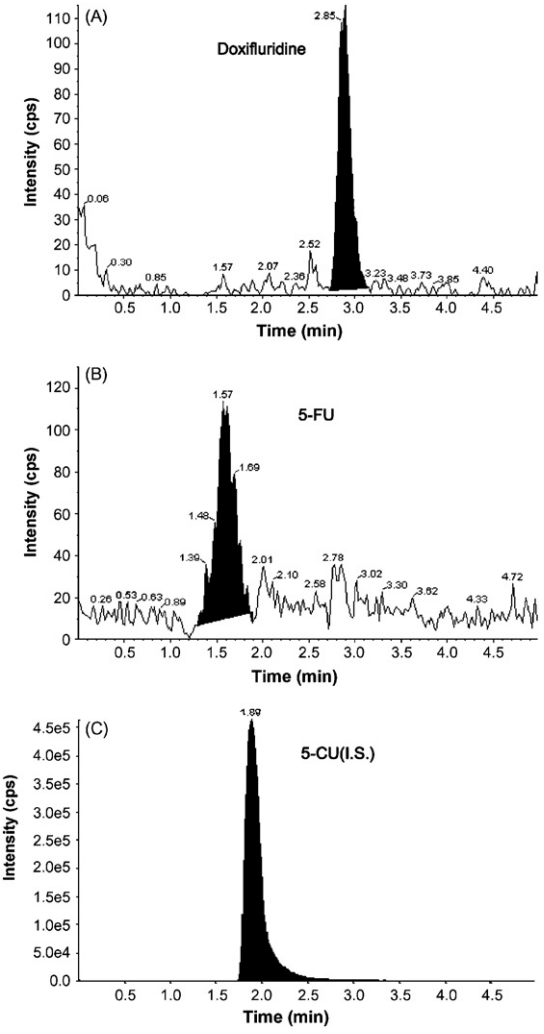

Fig. 2. LC/MS/MS chromatogram of (A) doxifluridine, (B) 5-FU and (C) 5-CU at LLOQ spiked into serum.

Table 1
Accuracy and precision for LLOQ samples.

Run no.	Doxifluridine (10 ng/mL)	5-FU (10 ng/mL)
1	9.8	9.8
2	10.1	11.5
3	9.1	9.2
4	10.6	11.4
5	9.2	10.5
Accuracy (%)	97.6	104.8
S.D.	0.6	1.0
CV (%)	6.4	9.5

obtained from three different calibration curves of doxifluridine and 5-FU. The back-calculated calibration standard points showed CVs ranging from 1.1 to 7.6% for doxifluridine and from 1.4 to 9.5% for 5-FU, respectively.

3.4. Intra-day accuracy and precision

QC samples at three concentrations were analyzed in six replicates for determining the intra-assay accuracy and precision of this method. The accuracy for doxifluridine and 5-FU ranged from 99.6 to 102.6% and from 100.3 to 102.0%, respectively with the precision (CV%) between 2.6 and 3.8% for doxifluridine, and between 0.9 and 4.2% for 5-FU, respectively (Table 3).

3.5. Inter-day accuracy and precision

The inter-assay accuracy and precision were calculated from six QC replicates at three concentrations for each compound on three days. The accuracy for doxifluridine and 5-FU ranged from 99.7 to 101.0% and from 99.3 to 101.1%, respectively with the precision (CV%) between 4.0 and 7.4% for doxifluridine, and between 1.7 and 5.1% for 5-FU, respectively (Table 3).

3.6. Stability

Analyte stability was investigated for a variety of conditions that were utilized for handling and storage of both standards and samples. Stability was confirmed if the average values of measured analyte concentrations were within 15% of their respective spiked concentrations. Stock solution stability of doxifluridine, 5-FU, and internal standard, 5-CU at concentration 100 μg/mL was investigated for 24 h at room temperature. All three analytes were shown to be stable with ranging from 95.5 to 106.0%. Freeze/thaw cycles were also evaluated to assure that no analyte losses occurred during sample freezing and thawing that were required for analysis and possible sample retests. The stability of doxifluridine and 5-FU in auto-sampler at 4 °C after sample preparation was evaluated. Results of the stability showed that there was no significant loss under the designed condition as listed in Table 4.

Short-term storage of serum at room temperature was investigated for some possible exposure to room temperature during any delays in the sample preparation process. The stability of the serum samples for 24 h at room temperature was evaluated after spiked with doxifluridine and 5-FU into serum. The concentration of doxifluridine was reduced to 38.2–65.4% ($P < 0.001$, paired t-test) for 24 h. Metabolism of doxifluridine into 5-FU or subtypes occurs in blood serum due to the activity of TP (thymidine phosphorylase) in blood might be one of the reason.

It has been reported in the previous study the plasma thymidine was temperature sensitive due to the high activity of TP and plasma thymidine concentration decreased significantly after 3 h at 23 °C [14]. They also reported that 2-deoxyuridine in plasma has significant loss at 23 °C [15].

At present study, the changes of doxifluridine and 5-FU based on time and temperature were investigated after spiking doxifluridine and 5-FU each into serum. The short-term stability of doxifluridine and 5-FU in serum kept on-ice, at 20 °C and 37 °C during 4 h is shown in Fig. 3. Every point was averaged from three measurements. The starting concentration was 400 ng/mL for both compounds. The relative changes were within ±15% for 4 h. Serum doxifluridine and 5-FU was stable for 4 h, regardless of temperature condition.

Our results showed that doxifluridine, similar to thymidine and 2-deoxyuridine, after spiked into serum decreased at room temperature during 24 h. The activity of TP in blood might be related to the decrease of doxifluridine. Therefore, sample preparation for real sample analysis should be performed within 4 h.

3.7. Recovery and matrix effect

The recovery was estimated by comparing the mean peak areas of the analytes in the extracted QC samples at concentrations of 30, 400, and 1600 ng/mL in six replicates with those obtained from the extracted blank serum samples post-spiked with corresponding neat solutions in six replicates. The overall recovery for doxifluridine and 5-FU was 87.1 and 77.7%, with the precision (CV%) of 12.4 and 11.4%, respectively.

The matrix effect of doxifluridine and 5-FU was assessed by comparing the peak area of the analytes in water (A), and that of standard spiked after sample preparation of blank plasma (B) to show that there was any ion enhancement or suppression during analysis. The samples at concentrations of 30, 400, and 1600 ng/mL

Table 2
Accuracy and precision for calibration curve samples of (a) doxifluridine and (b) 5-FU.

Concentration (ng/mL)	Measured ($n = 3$)	S.D.	Accuracy (%)	CV (%)
(a) Doxifluridine				
10	9.8	0.6	98.0	6.1
20	19.1	1.1	95.5	5.8
50	50.3	3.5	100.6	7.0
100	104.6	8.0	104.6	7.6
200	204.0	7.2	102.0	3.5
500	515.8	8.5	103.2	1.6
1000	1014.0	11.4	101.4	1.1
2000	1912.0	53.1	95.6	2.8
Concentration (ng/mL)	Measured ($n = 3$)	S.D.	Accuracy (%)	CV (%)
(b) 5-FU				
10	10.5	1.0	105.0	9.5
20	20.4	0.9	102.0	4.4
50	49.7	4.4	99.4	8.9
100	100.6	3.2	100.6	3.2
200	192.4	2.7	96.2	1.4
500	508.2	17.9	101.6	3.5
1000	1009.4	20.0	100.9	2.0
2000	1994.0	33.6	99.7	1.7

Table 3
Intra- and inter-accuracy and precision for doxifluridine and 5-FU in serum.

Analytes	Concentration (ng/mL)	Intra-day ($n = 6$)		Inter-day ($n = 6$)	
		Accuracy (%)	CV (%)	Accuracy (%)	CV (%)
Doxifluridine	30	99.6	2.6	100.6	7.4
	400	102.2	3.0	99.7	4.0
	1600	102.6	3.8	101.0	4.4
5-FU	30	100.3	4.2	99.3	5.1
	400	100.8	0.9	101.1	1.7
	1600	102.0	1.9	100.9	2.6

Table 4
Stability of doxifluridine and 5-FU in serum.

Storage condition	Relative concentration (%)					
	Doxifluridine (ng/mL)			5-FU (ng/mL)		
	30	400	1600	30	400	1600
Freeze–thaw, three cycles (n = 3)	101.0	97.7	101.3	95.5	100.4	100.3
Post-preparative, 24 h at 4 °C (n = 3)	93.8	101.6	98.0	96.5	101.0	99.7
Short-term stability 24 h at 20 °C (n = 3)	38.2	65.3	65.4	111.0	115.6	114.3

in six replicates showed 84.1 and 24.5% matrix effect, for doxifluridine and 5-FU with the precision (CV) of 12.8 and 9.6%, respectively. There was ion suppression for both doxifluridine and 5-FU, during analysis. The degree of ion suppression for two analytes was different, originating from different competition between an analyte and the coeluting, undetected matrix components reaction with primary ion formed in the LC–MS/MS interface. In spite of strong ion suppression of 5-FU during analysis, the precision (CV) for the matrix effect showed that the ion suppression was evenly effected during analysis. This overall results including recovery show that the developed liquid/liquid extraction using ethyl acetate/*iso*-propyl alcohol (9/1) solvent system is very effective to extract both doxifluridine and 5-FU, simultaneously.

The polarity for doxifluridine and 5-FU is different. The log *P* for doxifluridine and 5-FU is −1.40 and −0.89, respectively. Doxifluridine is much more soluble in water than 5-FU. For those materials, this developed solvent system gave good recovery rate and reliable extraction rate. Various solvent mixing ratios were investigated to develop effective solvent system for two materials with different polarity. Relatively strong polar organic solvent combination such as ethyl acetate and *i*-propyl alcohol were applied. The proper mixing ratio was empirically found to provide good recovery. As a result, ethyl acetate/*iso*-propyl alcohol (9/1) solvent system was found to be very effective for simultaneous sample preparation for both doxifluridine and 5-FU.

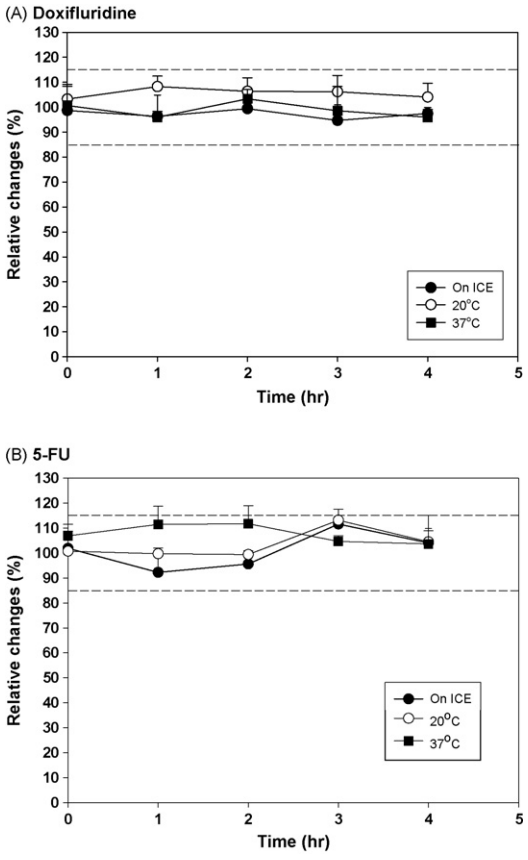

Fig. 3. Short-term stability on ice, at 20 °C, and at 37 °C for 4 h (A) doxifluridine and (B)5-FU.

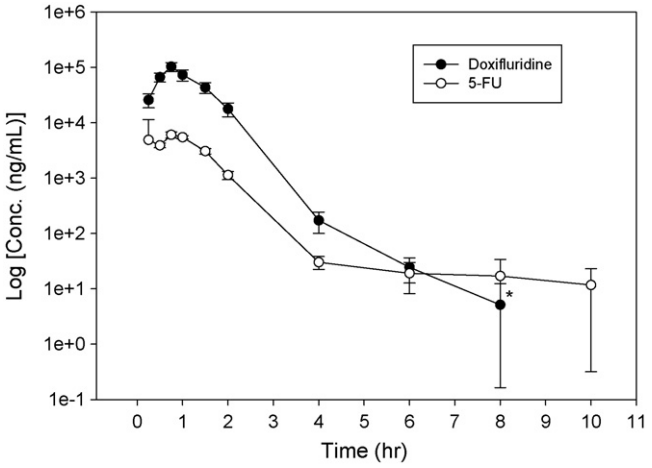

Fig. 4. Mean serum concentration–time profile in three monkeys after administration of single 100 mg/kg dose of doxifluridine. (*: below LLOQ at 10hr for doxifluridine)

3.8. Toxicokinetic data

The present LC–MS/MS method was used to measure serum concentrations of doxifluridine and 5-FU in monkeys following oral administration of doxifluridine at 100 mg/kg. The monkey blood samples were collected before administration and at 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8 and 10 h after oral administration of doxifluridine at 100 mg/kg from three monkeys. The mean serum concentration–time profile of doxifluridine and 5-FU is shown in Fig. 4. The suitability of the method was demonstrated using QC samples in every batch with good accuracy and precision during the analysis of study samples.

4. Conclusions

A bioanalytical method for the simultaneous determination of doxifluridine and 5-FU in monkey serum was developed. The selectivity, linearity, accuracy, precision and stability of the method were validated. This method provides simpler sample preparation procedure and very shorter LC running time than previously reported with high sensitivity and good reliability. Our results demonstrate that doxifluridine in serum is not stable in blood for 24 h. The stability was validated only for 4 h. Analytical samples should be prepared and analyzed within 4 h.

The method for the quantification of doxifluridine and 5-FU in monkey serum was successfully applied to the analysis of toxicokinetic samples. The reliability of the method was well established for real samples. Assay accuracy and precision were monitored through

QC samples and showed good reliability for both doxifluridine and 5-FU. Examples of TK results demonstrate the utility of this method. The present method is useful for pharmacokinetic and toxicokinetic studies of doxifluridine and 5-FU.

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