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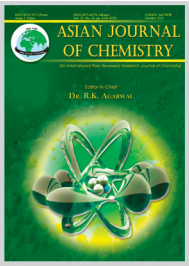

Quantitation of Floxuridine in K_2 EDTA Rat Plasma using HPLC Coupled with Tandem Mass Spectrometry

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A rapid, sensitive and specific method based on high performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) detection with electro spray ionization in negative ionization mode has been developed for the determination of floxuridine in K_2 EDTA rat plasma. Sample preparation involved liquid-liquid extraction with ethyl acetate. Chromatographic separation was performed on Agilent Eclipse XDB column (C_{18} 150 × 4.6 mm 5 µ), with a mobile phase consisting of methanol and 1 mM ammonium acetate at a flow rate of 0.4 mL/min. The analytes were then detected by monitoring the transitions m/z 245.2-155.1 for floxuridine and m/z 321.5-143.9 for 5-bromouridine(IS) using API-4000 LC-MS/MS system (applied biosystems). Calibration curves were linear within the range of 0.050 to 50.00 ng/mL. The lower limit of quantitation (LLOQ) was 0.050 ng/mL. The method has been fully validated in rat plasma. This method can be successfully applied to the pharmacokinetic study sample analysis.

Key Words: Floxuridine, LC-MS/MS, Pharmacokinetic.

INTRODUCTION

Floxuridine, a pyrimidine antagonist is a nucleoside consisting of the pyrimidine base fluorouracil and the sugar[1,2]. The effects of floxuridine in carcinomas principally confined to the peritoneal cavity have been studied[3,4]. The advantage of this route of administration is that it allows for increased drug expose to the tumor for prolonged time periods prior to drug metabolism by the liver[5]. In contrast, unless there is significant metabolism of floxuridine to fluorouracil. Floxuridine acts primarily by inhibiting thymidylate synthetase.

Measurement of plasma levels and peritonea levels of floxuridine and the evaluations of pharmacokinetic data may provide a better understanding of local and systemic toxicity of floxuridine.

Presently available high performance liquid chromatography (HPLC) methods for measuring floxuridine[6,7] lack sensitivity, more plasma volume and required more time for analysis. The current methodology describes sensitive LC-MS/MS method with an lower limit of quantification of 0.050 ng/mL in 200 µL of plasma volume for measuring floxuridine.

EXPERIMENTAL

5-Fluoro-2'deoxyuridine (floxuridine), 5-bromouridine was purchased from Sigma Aldrich India. High performance liquid chromatography grade deionized water, methanol, ammonia, ethyl acetate were obtained from aualigens and ammonium acetate were obtained from Sigma.

Stock and working solutions preparations: For stock solutions, all compounds were dissolved in methanol and stored at 2-8 ºC.

The calibration curve standards were prepared in 50:50 (water:methanol) by adding known amounts of floxuridine stock solutions. Lower limit of quantification (LLOQ) quality control and low-quality control (LQC) samples were obtained by spiking floxuridine in plasma; the final concentrations were 0.05 and 0.15 ng/mL, respectively. Middle-quality control (MQC) and high-quality control (HQC) samples were obtained by spiking in plasma with concentrations of 25 and 40 ng/mL, respectively. All calibration curves consisted of one blank sample and ten calibration points in the concentration range of 0.05-50.024 ng/mL. The concentrations were corrected for potency and amount weighed. The resulting peak area ratios were plotted against the concentrations.

Sample preparation: 10 µL of working standard solution (floxuridine) is spiked to190 µL drug-free SD rat plasma and aliquot 25 µL of spiked plasma to this 25 µL of internal standard (IS) working solution (5-bromouridine) 40 µg/mL and 50 µL of 1 mM ammonium acetate were added and were mixed thoroughly by vortexing for 10 sec. 500 µL of ethyl acetate

was added as an extraction solvent. After vortex-mixing for 2 min, the samples were centrifuged at 14000 rpm for 10 min; the organic layer was separated and evaporate to dryness under nitrogen gas at 55 ºC. The residue was dissolved in 150 µL of reconstitution solution (50:50 v/v 1 mM ammonium acetate: methanol) and 5 µL was injected into HPLC system.

High performance liquid chromatography (HPLC) and mass spectrometer conditions: The high performance liquid chromatography system consist of a Shimadzu Prominence model equipped with prominence binary pump, SIL HTC auto injector, CTO oven coupled with mass detector API-4000 (applied Biosystems) triple Quadra pole system. Separation was achieved by using Agilent Eclipse XDB C_{18} 150 × 4.6 mm; 5 µ column. The mobile phase contains 1 mM ammonium acetate and methanol. The flow rate was set to 0.4 mL/min with gradient mobile phase starting 90 % aqueous till 2 min and from 2.01 min increase the organic to 70 % till 5 min and after 5.01 min increased the aqueous to 90 % until the end of chromatogram. The total run time was 6 min.

Electron spray ionization source was used for mass spectrometric analysis and detection. Mass spectrometric analysis was performed in negative ion mode and set up in multiple reaction monitoring (MRM). Nitrogen was used as a current gas (40 psi). Zero air used as collision gas for fragmentation of the parent molecule. The product ions for floxuridine m/z 245.2-155.1 and for 5-bromouridine m/z 321.5-143.9.

Selectivity and specificity: Blank K_2 EDTA rat plasma samples from six different individual lots were processed and analyzed without addition of analyte and internal standard (BLK) Figs. 1 and 2. In addition, each lot was spiked separately with six replicates of floxuridine at the lower limit of quantification and internal standard. Significant matrix interference (> 20 % of the analyte peak area of the lower limit of quantification sample, > 5 % of the internal standard peak area of the blank matrix) was not observed in six out of six blank samples at the retention time of analyte or the internal standard.

Recovery: The recovery exercise was performed at all quality control levels by comparing the response (area) of processed quality control samples with those of directly injected quality control samples.

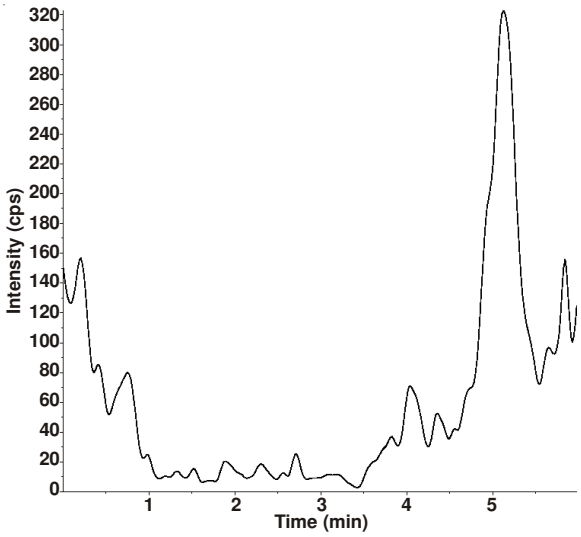

Fig. 1. Chromatogram for floxuridine in rat blank plasma extract

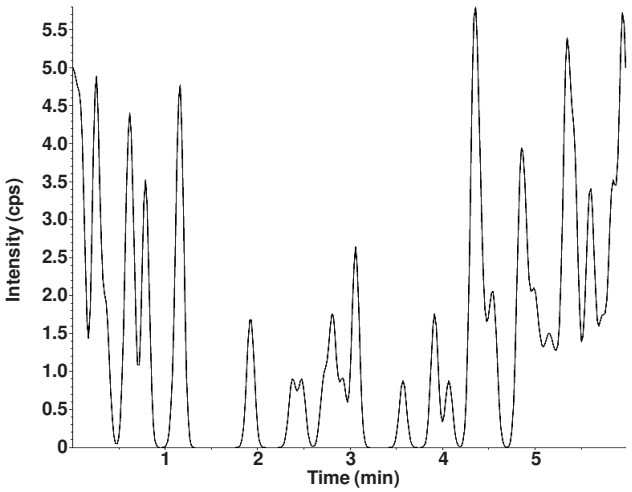

Fig. 2. Chromatogram for 5-bromouridine in rat blank plasma extract

Dilution integrity: The DQC solution of analyte was spiked in rat plasma. From that 1/5th and 1/10th dilutions were prepared. From each dilution, six aliquots were processed and analyzed along with calibration curve samples. Spiked concentration of DQC sample for floxuridine was 187.590 µg/mL.

Intraassay and interassay precision and accuracy: Intraassay precision and accuracy were evaluated using replicates (N = 6) from each of the three quality control concentrations from the first precision accuracy batch. Interassay precision and accuracy were assessed on three validation batches

Stability: The stability of floxuridine was studied in rat plasma at room temperature (bench top) for 4 h. The bulk-spiked plasma and water samples stored at -50 ºC underwent three freeze-thaw cycles. In addition, a long-term (30 days) stability study was done in rat plasma stored at -50 ºC.

RESULTS AND DISCUSSION

A high-performance liquid chromatographic mass spectrometric method for the estimation of floxuridine in rat plasma has been developed and validated according to the principles of good laboratory practices. The floxuridine was validated over a concentration range of 0.050 to 50.024 ng/mL. Sample cleanup was accomplished by liquid-liquid extraction using ethyl acetate. The reconstituted samples were analyzed by LC-MS/MS using a Agilent Eclipse XDB column (C_{18} 150 × 4.6 mm 5 µ). The retention times of floxuridine and 5-bromouridine were between 4.0 and 4.3 min, with a total run time of 6 min.

The lower limit of quantitation was 0.05 ng/mL for floxuridine. The between-run precision and accuracy for floxuridine at 0.05 ng/mL were 2.8 % and 102 %, respectively. (Table-1) The linearity of the method was determined by a weighted least-squares regression analysis of an eight point standard curve. The calibration lines were shown to be linear from 0.050 to 50.024 ng/mL. Best-fit calibration lines of the ratio of floxuridine to internal standard (IS) peak area versus the concentration of calibration standards were determined by least-squares regression analysis with weighting factors of $1/x^2$. The r^2 values were consistently > 0.99 during the course of validation.

TABLE-1
INTRAASSAY PRECISION AND ACCURACY OF FLOXURIDINE FOR QUALITY CONTROL SAMPLES

Floxuridine concentration (ng/mL)	Intra day		Inter day	
	CV (%)	Accuracy	CV (%)	Accuracy
0.150	5.5	99.7	5.1	100.3
3.700	2.2	107.2	4.2	103.9
40.500	1.9	95.6	3.5	93.8

The precision of the assay was measured by the percentage coefficient of variation over the concentration range of lower limit of quantification, low-quality control, middle-quality control and high-quality control samples during the course of validation. The accuracy of the assay was defined as the absolute value of the ratio of the calculated mean values of the quality control samples to their respective nominal values, expressed as percentages. Within-batch precision ranged from 1.9 to 5.5 % and within-batch accuracy ranged from 95.6 to 107.2 %. Intraassay precision ranged from 3.1 % to 5.1 % and intraassay accuracy ranged from 93.8 to 100.3 % (Table-1).

Inter-assay precision ranged from 0.0 to 9.1 % and interassay accuracy ranged from 88.1 to 105.6 % (Table-2).

TABLE-2
INTERASSAY PRECISION AND ACCURACY OF FLOXURIDINE FOR STANDARD CURVE SAMPLES

Floxuridine concentration (ng/mL)	Inter day	
	CV (%)	Accuracy (%)
0.050	2.8	102.0
0.200	5.8	102.8
0.500	2.1	101.7
1.000	9.1	105.6
5.002	8.2	103.6
10.005	5.3	100.0
20.010	0.0	99.7
50.024	2.1	88.1

The mean stability of floxuridine in rat plasma ranged from 95.4 to 101.0 % and 88.8 to 91.9 % for one and three freeze-thaw cycles, respectively during bench-top stability analysis, floxuridine was found to be stable up to 4 h and the mean stability ranged from 89.1 to 101 %. Floxuridine was found to be stable for up to 30 days of storage (plasma) below -50 ºC and the mean stability ranged from 92.7 to 101.2 % (Table-3). The absolute recovery of floxuridine and internal standard was calculated for replicate spiked quality control samples (low-quality control, middle-quality control and high-quality control). Results indicate overall recoveries of 36.86 % for floxuridine and 43.8 % for internal standard.

TABLE-3
LONG TERM STABILITY OF FLOXURIDINE AT –50 ºC

Floxuridine concentration (ng/mL)	Long term stability at –50 ºC				
	Stability		Comparison		
	CV (%)	Accuracy	CV (%)	Accuracy	Change (%)
0.150	4.2	101.2	5.5	99.7	-1.6
40.500	2.4	92.7	1.9	95.7	3.1

The results demonstrate acceptable dilution integrity for 1/5th and 1/10th times. Within-batch precision and accuracy for five times dilution were 3.2 % and 92.2 %, respectively, whereas within-batch precision and accuracy for four times dilution were 6.6 % and 91.2 %, respectively.

In this study, a reverse-phase HPLC method with mass spectrometric detection using 5-bromouridine as an internal standard was developed. Various combinations of organic and aqueous phases were tried and better chromatography with lower baseline was achieved using 1 mM ammonium acetate and methanol (50:50, v/v) as the mobile phase. Response was observed and the flow rate was optimized to 0.4 mL/min.

The extraction of floxuridine is done by liquid-liquid extraction procedure. Both precipitation and solid-phase extraction procedures were assessed initially for the extraction of floxuridine from plasma. Better sample cleanup, reproducibility and cost effective were obtained by liquid-liquid extraction. The method described is sensitive, selective, precise and accurate for the determination of floxuridine in rat plasma at very low concentrations (< 0.1 ng/mL) over a concentration range extending up to 50 ng/mL.

Conclusion

A simple, sufficiently sensitive and reproducible method was developed for the quantification of floxuridine in plasma. The stability studies demonstrated that floxuridine was stable during normal assay procedures and in long-term frozen storage conditions (below -50 ºC). This should allow clinical samples to be stored and analyzed efficiently. The main advantage is improved sensitivity that allows the quantification of 0.050 ng/mL of floxuridine in plasma samples.

This assay can be successfully applied to investigate floxuridine in rat plasma, human plasma and Homogenized tissue tumor samples of various pharmacokinetic investigations, therapeutic drug monitoring in cancer patients from clinical studies and toxicokinetic studies in pre clinical studies. Rat is an adequate model for oncology studies. It has been used extensively to study the tumor growth and therapeutic drug concentrations in plasma. So the method is validated in rat plasma.

REFERENCES

1. United States Pharmacopeial Convention, Rockville, MD, pp. 428, 583 (1985).
2. L.A. Trissel, In: Handbook on Injectable Drugs, American Society of Hospital Pharmacists, Bethesda, MD, edn. 4, pp. 247, 323 (1986).
3. E. Mini, B.A. Moroson and J.R. Bertino, *Cancer Treat. Rep.*, **71**, 381 (1987).
4. R.F. Ozols, T.E. Speyer, J. Jemkins and C.E. Myers, *Cancer Treat. Rep.*, **68**, 1229 (1985).
5. R.L. Dedrik, C.E. Mayers, P.M. Bungay and V.T. Devita, *Cancer Treat. Rep.*, **62**, 1 (1978).
6. J.A. Smith-Rogers, W.P. Tong, M.E. Duafala, M. Markman and J.R. Bertino, *J. Chromatogr.*, **566**, 147 (1991).
7. Z. Y. Ma, L. L. Tu, W. J. Li and X. Y. Peng, *Yaowu Fenxi Zazhi*, **24**, 550 (2004).