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UPLC method Validation for bioanalysis of Gemcitabine in rat PK study using VAMS methodology (Volumetric Absorptive Microsampling)

HAFIS JAMAL AYYIL[1], S. KATHIRVEL [1] & RAJA RAJESWARI K*

[1]National College of Pharmacy, KMCT Group of Institutions, Manassery, Kozhikode-673602, Kerala, India.

*Department of Pharmaceutical Analysis, Sri Sivani College of Pharmacy, Srikakulam-532 402, Andhra Pradesh, India.

***Address for Correspondance:**

Dr. Raja Rajeswari Katta,

Professor & HOD,

Department of Pharmaceutical Analysis,

Sri Sivani College of Pharmacy,

Chilakapalem Junction,

Srikakulam-532 402,

Andhra Pradesh, India

ABSTRACT

Volumetric absorptive microsampling (VAMS) is a simple intuitive technique for collecting and quantitative analysis of dried blood samples. It enables the collection of an accurate blood volume regardless of blood hematocrit. A bioanalytical method for the determination of gemcitabine in dried blood supported on VAMS samplers has been validated and used to support a pharmacokinetic study in rat. The calculated PK parameters were comparable to those obtained from blood–water (1:1, v/v) samples. VAMS is demonstrated to be a robust method that simplifies both the blood sample collection and bioanalytical laboratory procedures and generates

high quality quantitative data. Waters Acquity UPLC system using an Acquity BEH C18 column (100x2.1 mm, 1.7 µm) was used for chromatographic separation by isocratic elution using acetonitrile-water (35-65) as the mobile phase at a flow rate of 0.5 mL/min. Gemcitabine was administered to rat orally at 3 mg/kg for conducting the PK study and the blood was collected at various time intervals using VAMS sampler which consists of a hydrophilic polymeric tip, absorbs an accurate sample volume within 2–4 s by wicking, attached to a molded plastic handle. The tip is white before use and turns completely red when filled with blood, and the blood samples were processed after collection and analyzed by UPLC. The intra-day and inter-day accuracy of gemcitabine were 93.6–108.7% and 94.2–111.5% respectively, and the precision (RSD, %) was less than 15% for both intra-day and inter-day measurements. Gemcitabine has a good linear relationship in the range of 50-500 ng/mL with r2 value of 0.997. A robust and reliable UPLC method was fully optimized and developed to detect the blood concentration of gemcitabine in rats and the samples were analyzed by Empower software.

KEYWORDS: Gemcitabine, VAMS, UPLC, Validation, Bioanalytical, Pharmacokinetics

INTRODUCTION

Gemcitabine is a synthetic pyrimidine nucleoside prodrug, a nucleoside analog in which the hydrogen atoms on the 2' carbon of deoxycytidine are replaced by fluorine atoms[1]. This drug treats cancers including testicular cancer, breast cancer, ovarian cancer, non-small cell lung cancer, pancreatic cancer, and bladder cancer[2]. Gemcitabine is in the nucleoside analog family of medication. It works by blocking the creation of new DNA, which results in cell death[3]. Gemcitabine can increase your risk of bleeding or infection[4]. Even though the drug is approved for medical use in 1995, very less clinical data is available for this and no rapid UPLC method has been reported so far to analyse the bio samples. When gemcitabine is incorporated into DNA it allows a native, or normal, nucleoside base to be added next to it. This leads to "masked chain termination" because gemcitabine is a "faulty" base, but due to its neighbouring native nucleoside it eludes the cell's normal repair system (base-excision repair). Thus, incorporation of gemcitabine into the cell's DNA creates an irreparable error that leads to inhibition of further DNA synthesis, and thereby leading to cell death. The form of gemcitabine with two phosphates attached (dFdCDP) also has activity; it inhibits the enzyme ribonucleotide reductase (RNR), which is needed to create new DNA nucleotides. The lack of nucleotides drives the cell to uptake more of the components it needs to make nucleotides from outside the cell, which also increases uptake of gemcitabine. Gemcitabine is marketed as Gemzar and it is available as intravenous injection. It is approved by the FDA to treat advanced ovarian cancer in combination with carboplatin, metastatic breast cancer in combination with paclitaxel, non-small cell lung cancer

in combination with cisplatin, and pancreatic cancer as monotherapy. It is also being investigated in other cancer and tumour types. The aim of the current research is to develop and validate a rapid, reliable, sensitive and simple ultra-performance liquid chromatography method for the quantification of Gemcitabine in whole human blood by Volumetric Absorptive microsampling (VAMS)[5] technique. The advantages of taking microsamples (typically blood samples within the range 10–100 µL), particularly for the determination of rodent pharmacokinetics (PK) and toxicokinetics (TK) has been well documented[6]. The VAMS sampler consists of an absorbent tip, that wicks up an accurate volume of blood (approximately 10 µL), attached to a plastic handle. The volume of blood absorbed is independent of the HCT of the blood. The sample collection procedure involves dipping the tip of the sampler into a pool of blood, for 4–6 s. The sample that is collected is then in the format used for storage and shipping, with only drying and packaging required as additional processing steps. In addition, since the sampling device itself becomes the sample to be analyzed, there is also a reduction in the workflow complexity in the bioanalytical laboratory, with the elimination of the need for aliquotting as with liquid samples, or sub-punching of DBS samples[7,8]. Further, the design of the sampling device readily enables automation using standard liquid handling robots.

A very few analytical methods are available for the determination of Gemcitabine by chromatographic methods. Although several HPLC[9-16] and LC-MS[17-24] methods are available for bioanalysis but most of them are very expensive and time consuming. Till date there is no UPLC method reported for bioanalysis of Gemcitabine.

The objective of the present work is to develop and validate a simple assay on UPLC (Ultra Performance Liquid Chromatography) using VAMS technique to determine Gemcitabine concentrations in whole human blood. The developed bioassay is validated using internationally accepted criteria. After complete validation, the method was applied to analyze study sample analysis in rats by giving a single oral dose at 3 mg/kg body weight. Data generated from dried VAMS samples is compared to that from VAMS samples extracted before drying and that from the more conventional approach of blood sampling, where whole blood is quantitatively diluted with water. In addition, the effect of HCT, storage and initial blood temperature are investigated.

EXPERIMENTAL

Instrumentation and Chromatographic Conditions

UPLC–UV Analysis

The LC system consisted of a Waters Acquity UPLC with Empower software equipped with a photodiode array detector. A Acquity BEH C_{18} column (100x2.1 mm, 1.7 μm particle size) from Waters was used as stationary phase and temperature maintained at 20°C. The mobile phase consisted of Acetonitrile and water (35:65) in isocratic mode pumped at a flow rate of 0.5 mL/min. Analysis was performed for 5 min at the detection wavelength of 275 nm and the injection volume was 5 µL. The autosampler maintained at 4°C

Chemicals

Gemcitabine and internal standard (2'-deoxycytidine) are purchased from Sigma–Aldrich Trading Co., Ltd. (Shanghai, China). Acetonitrile and methanol of HPLC grade and all other chemicals were obtained from Merck (Mumbai, India). Water used in the entire analysis was prepared from Milli-Q water purification system from Millipore (Milford, MA, USA). Biological matrix (whole human blood) was obtained from Vimta Labs (Hyderabad, India) and stored at −20°C until use.

Preparation of Calibrators and QC Samples

A standard stock solution of Gemcitabine was prepared by dissolving standard 50 mg of Gemcitabine into 50 ml volumetric flask, to this added 30 ml of methanol and sonicated for 10 minutes at a temperature not exceeding 20°C. Allowed the solution to attain room temperature and then diluted to the volume with methanol to have a solution with a concentration of 1000 µg/mL. Calibration standard and quality control (QC) samples were prepared by adding corresponding working solutions with drug-free human blood. A volume of 10 mL of appropriate diluted stock solution at different concentrations and 10 mL of IS at a fixed concentration were spiked into 200 µL of human blood to yield final concentrations of calibration samples 50, 100, 150, 200, 250, 300, 400 and 500 ng/mL. The final concentration of IS was 100 ng/mL. Similarly, QC samples were prepared at four concentration levels LLOQ (50 ng/mL), LQC (150 ng/mL) MQC (250 ng/mL) and HQC (400 ng/mL) in a similar manner to the calibration standards but from an independent stock solution.

Sample preparation

Analytes were extracted from blood by employing VAMS method, vortexed for 1min and then centrifuged at 10,000 rotations per minute for 10 min on refrigerated centrifuge at 4°C. The supernatant layer was separated and filtered through 0.45 µm syringe filters and 10 µL of the solution was injected for UPLC analysis.

The newer sampling technique, Volumetric Absorptive Microsampling (VAMS) allows reduction of volume from milliliter to microliter (sample volume ~ 10µl). The micro sampling devices (Mitra®) have overcome almost all drawbacks of conventional sampling with a few additional benefits. A novel dried blood sampler, VAMS, allows consistent blood volume regardless of Hematocrit (Hct). It is available in a configuration of 2 samples with volume 10, 20 and 30 µl. A sampler of 10 and 20 µl is usually used for sampling in animals and 30 µl in humans. The unique device consists of an absorbent polymeric tip which enables the collection of fixed, a small volume of blood by capillary action. The sample is obtained either by finger or heel prick for humans and tail vein in rodents. During collection, the sampler is filled by holding the handle at an angle of 45° and dipping only the tip into blood drop and allowing it to fill. The tip of the sampler should not be completely plunged into the blood sampler. This may cause overfilling of the sample. The device is self-indicating i.e. when the tip is filled, it turns red. The tip is attached to a handle, which is designed in a way that prevents the sampler tip coming into contact with surfaces during storage and shipping. Samples can be shipped or stored at room temperature. VAMS device ensures the homogeneity of the sample, as a precise volume is absorbed on to the tip. During sample preparation, either the tip is removed from the handler or the whole device is used. This device enables ease of sample pretreatment as the centrifugation step of the liquid matrix and sub-punching step of DBS (Dried blood spot) is subtracted. Moreover, the sampler is configured to fit in manual or automated extraction devices. The greatest advantage of VAMS over DBS is that VAMS enables the precise and accurate collection of blood volumes for quantitative bioanalysis. The dried VAMS calibration and QC samples were extracted by removing the tip from its sampler by pulling the tip against the inside of the extraction tube, to which 200 µL of methanol containing internal standard was added. The sealed tubes were mixed on a lateral shaker for an hour. The extracts were diluted 9-fold with methanol–water (1:1, v/v), prior to analysis for gemcitabine by UPLC.

Preparation and extraction of wet samples from VAMS samplers

In order to prepare wet VAMS samples, blood was absorbed onto the VAMS tip as previously described, and then immediately removed from the holder by pulling the tip against the side of a 1.4 mL Micronic tube to which water (100 µL) had been added. After sealing, the tube was vortex mixed and allowed to stand for 1 h to allow cell lyses to occur. The wet VAMS blood–water samples were either used immediately, or stored frozen at -20◦C. Gemcitabine was extracted from aliquots of the wet VAMS blood–water samples by protein precipitation,

following the addition of 5 volumes of methanol containing internal standard (5 µg/mL) and EDTA, followed by centrifugation at 5000 rpm at 4◦C for 10 min. The supernatant was diluted 2-fold with methanol-water (1:1, v/v) prior to analysis by UPLC.

Preparation and extraction of blood–water (1:1, v/v) samples

Blood–water samples were prepared by mixing equal volumes of blood and water (100 µL of each) and allowing them to stand for an hour. These were either used immediately, or stored frozen at -20◦C. The extraction procedure for blood–water samples was the same as for wet VAMS samples, except 10 volumes of methanol containing internal standard was used at the precipitation stage and the supernatant was diluted 9-fold with methanol-water (1:1, v/v) prior to analysis by UPLC.

Analytical Validation

All validation experiments were performed according to the Bioanalytical Method Validation Guidance for Industry[25] and the ICH guidelines[26] on validation of bioanalytical methods.

Assay Specificity and Selectivity

Specificity was assessed by verifying the absence of significant interference in the biological control medium with regard to the retention time of the compound (s) to be assayed. The specificity of the method was confirmed by comparing chromatograms of blank matrix, spiked matrix with analyte at LOQ concentration. No interfering endogenous peaks were observed around the retention time.

Linearity

A calibration curve was prepared within the range of 50 to 500 ng/mL gemcitabine in each run. Half of the calibration samples were analyzed at the beginning of the run and half at the end. The simplest calibration model and weighting procedure were used. The calculations of the curve's parameters were based on the ratio of the peak areas of gemcitabine/IS versus the concentration of gemcitabine. Gemcitabine concentrations for samples were calculated from the curve's equation obtained by means of linear regression.

Accuracy of back-calculated calibration samples should be within ±15% of the corresponding nominal concentration, except at the lowest concentration level, where the accuracy should be within ±20%. Per calibration curve, a maximum of 33% of the calibration samples, except the LLOQ and upper limit of

quantification (ULOQ, 500 ng/mL), may differ from these specifications. At least 6 concentration levels were represented in each curve.

Matrix Effect, Extraction Recovery, and Process Efficiency

The influence of the matrix on the quantification of Gemcitabine was monitored using a comparison of: (1) the instrument response for the low, medium, and high QCs (n = 4 per level) injected directly in mobile phase (neat solutions), (2) the same amount of analyte added to extracted blank samples (post extraction spiked samples), and (3) the same amount of analyte added to the biological matrix before extraction (pre extraction spiked samples). Total process efficiency was calculated from the ratio of mean peak areas of Gemcitabine in extracted validation samples versus neat unextracted samples. This term accounts for any loss in signal attributable to the extraction process or matrix effect. Extraction recovery was calculated from the ratio of mean peak areas of Gemcitabine in extracted validation samples versus blank samples spiked after extraction. The absolute matrix effect was calculated from the ratio of mean peak areas of Gemcitabine in blank samples spiked after extraction versus neat unextracted samples. If the ratio was 85% or 115%, an exogenous matrix effect was inferred.

Matrix Variability

To confirm that the biological matrix would not interfere with the assay, the selectivity of the developed method was tested by analyzing 6 different lots of blank blood samples and also 6 different lots of blank urine samples spiked with IS at the LLOQ level (n = 3 per lot), and blank blood samples with no IS (n = 3 per lot) against a calibration curve. The results for the LLOQ samples were considered acceptable if the precision from each matrix lot was ±20% and the accuracy was within the range of 80%–120%. The acceptance criterion for the analysis of the blank samples from the 6 individual lots was based on the raw peak areas found at the retention times of Gemcitabine and IS. No more than 10% of the blank samples could have peak areas greater than 20% of the average peak area of Gemcitabine in the LLOQ QCs.

Stability studies

Stability evaluations were performed in both aqueous and matrix based samples. Stability evaluations in matrix were performed against freshly spiked calibration standards using freshly prepared quality control samples (comparison samples). Gemcitabine stability in blood was evaluated by performing bench top stability, long-term stability, short term stability and freeze-thaw stability. The processed samples were studied for stability in auto sampler at 10°C. Stability in blood was evaluated at both low and high QC level by comparing the mean response ratio of stability samples against the comparison samples.

RESULTS AND DISCUSSION

Chromatographic and detection parameters

Optimal chromatographic conditions were obtained after running different mobile phases with a reversed-phase C18 column. The different columns tried were Symmetry C18, Luna C18 and Zorbax C18. The best results were observed with the Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 µm particle size) using acetonitrile and water (35:65) as mobile phase. Variation of the column temperature between 20 and 30°C did not cause significant change in the resolution, however changes in retention time were observed. The column was used at 20°C at a flow rate of 0.5 mL/min. The method allowed the separation of analyte with IS in 5 min (Figure-1) runtime.

Specificity, Linearity, Accuracy and Precision

The specificity of method was confirmed by comparing chromatograms of blank matrix, spiked matrix with analyte at LOQ concentration. No interfering endogenous peaks were observed around their retention times. The eight point calibration curve for the analyte showed a linear correlation between concentration and peak area. Calibration data (Table 1) indicated the linearity ($r^2 > 0.99$) of the detector response for all standard solutions from 50 to 500 ng/mL The limits of detection by UPLC was found to be 20 ng/mL and LOQ was found to be 50 ng/mL. All standards and samples were injected in triplicate. Multiple injections showed that the results are highly reproducible and showed low standard error. A recovery experiment was performed to confirm the accuracy of the method. Blank blood was spiked with Low QC, Mid QC and High QC levels of the standard stock solution and then extracted and analyzed under optimized conditions. The extraction recoveries of all samples from human blood were in the range of 93.7-112.4% with relative standard deviations less than 10.0%, which indicates the sample preparation technique is suitable for extracting (Table 2). Intra- and inter-day precision of the method was determined by analyzing QC samples on two consecutive days and the obtained intra-day accuracies were in the range of 93.6–108.7% and inter-day accuracies were in the range of 94.2–111.5%. The recovery results are displayed in Table 3 and Table 4. To investigate carry-over from one sample to the other in the autosampler, each validation run containing a calibration curve included a blank sample analyzed directly after the sample at the ULOQ calibration level. The response of interfering peak (s) in the blank sample should not exceed 20% of the response of the component peak at the LLOQ calibration sample concentration. To demonstrate that the method is suitable for blood sample with test compound concentration higher than the ULOQ, the dilution integrity was

assessed using validation samples spiked with the test compound at 2-, 4-, and 10-fold the concentration of the high QC. The dilution test was performed by increasing the concentration of IS by the appropriate dilution factor. After extraction, the dry extract was taken up with a volume of injection solvent also multiplied by the same factor. Accuracy of the calculated concentrations within the range of 85%–115% of the nominal values would suggest that samples containing Gemcitabine at a higher concentration than the ULOQ can be diluted using the above tested dilution method.

Stability evaluations were performed in both aqueous and matrix-based samples. The stock solutions were stable for a period of 24 h at room temperature and for 60 days at 1–10°C. Stability evaluations in matrix were performed against freshly spiked calibration standards using freshly prepared quality control samples (comparison samples). The processed samples were stable up to 36 h in auto sampler at 10°C. The long-term matrix stability was evaluated at −20°C over a period of 60 days. No significant degradation of analytes was observed over the stability duration and conditions. The long-term stability results presented in Table 5 were within 85–115%. Stability in blood was evaluated at both low and high QC level by comparing the mean response ratio of stability samples against the comparison samples. The short-term stability of analyte at room temperature was within 85–115% upto 24 h. The stability results presented in Table 6 and Table 7. Gemcitabine was stable upto 10 h on bench top at room temperature and over 3 freeze–thaw cycles. In human blood , the freeze-thaw study was carried out and the results are presented in Table 8 and Table 9. The variability of the matrix effect in whole human blood has resulted a very minute changes in the recovery of middle concentration of calibration curve. The results of Matrix effect area presented in Table 10.

Autosampler Carry-Over Test

To investigate carry-over from one sample to the other in the autosampler, each validation run containing a calibration curve included a blank sample analyzed directly after the sample at the ULOQ calibration level. The response of interfering peak (s) in the blank sample should not exceed 20% of the response of the component peak at the LLOQ calibration sample concentration.

Dilution Integrity Test

To demonstrate that the method is suitable for a blood sample with test compound concentration higher than the ULOQ, the dilution integrity was assessed using validation samples spiked with the test compound at 2-, 4-, and

10-fold the concentration of the high QC. The dilution test using blood samples was performed by increasing the concentration of IS by the appropriate dilution factor. After extraction, the dry extract was taken up with a volume of injection solvent also multiplied by the same factor. Accuracy of the calculated concentrations within the range of 85%–115% of the nominal values would suggest that a blood sample containing Gemcitabine at a higher concentration than the ULOQ can be diluted using the above tested dilution method.

Effect of blood temperature

The ruggedness of the assay to variations in the temperature of the blood used to prepare VAMS samples was assessed by comparing the bias of dried VAMS samples generated at low and high QC levels from pools of blood held at 4◦C, ambient temperature (25°C) and 37°C. The maximum bias observed, against a calibration line prepared at ambient temperature, was 11% and the maximum with-in run precision was 5.8% indicating that the temperature of the blood used to generate the samples did not influence the observed concentration. The effect of Hematocrit on the volume of blood absorbed was investigated on low QC (Figure-2) and high QC level (Figure-3) and proved to be promising over an acceptable range.

Application of the method to pharmacokinetic study in Rat

Wistar rats (220±20 g) used were maintained in a clean room at a temperature between 22±2°C with 12 h light/dark cycles and a relative humidity rate of 50±5%. Rats were housed in cages with a supply of normal laboratory feed with water ad libitum. For all of the studies, the animals (n=6) were deprived of food 12 h before dosing, but had free access to water. In order to verify the sensitivity and selectivity of the developed method in a real-time situation, the developed UPLC method was successfully applied to a pharmacokinetic study by administration of Gemcitabine as single solution to six male wistar rats by oral route using BD syringe attached with oral gavage needle (size 18) at the dose of 3 mg/kg body weight (Figure-4). Approximately, a few drops of blood, drawn by dipping the tips of VAMS samplers into the blood in such a way that the tip just broke the liquid surface. The tips took between 2 and 4 s to completely absorb the blood and fill with color, depending upon the HCT of the blood and the depth to which they were immersed. Although the tip was considered full when it had completely colored, it was held for an additional 2 s in the blood pool before being removed and dried. Care was taken during the filling process to ensure that tips were not submerged past the shoulder. The VAMS samples were dried for a minimum of two hours, in freely circulating laboratory air (21°C, 55% relative humidity,

controlled but not monitored) in such a way that the tips did not touch each other or their surroundings. The VAMS samples were extracted by removing the tip from its sampler by pulling the tip against the inside of the extraction tube, to which 200 µL of methanol containing internal standard (100 ng/mL) was added. The sealed tubes were mixed on a lateral shaker for an hour**.** The extracts were diluted with methanol–water and centrifuged in diluent at 10,000 rpm for 10 min. The obtained supernatant samples were transferred into pre-labeled micro vials. The time intervals for the sample collection were 0 (predose), 0.5, 1, 2, 4, 6, 8, 12, 24 and 48 h (postdose).

The blood samples thus obtained were stored at –30°C till analysis. Post analysis the pharmacokinetic parameters were computed using WinNonlin® software version 5.2 and SAS® software version 9.2.

The pharmacokinetic parameters evaluated were Cmax (maximum observed drug concentration during the study), AUC_{0-48}(area under the blood concentration–time curve measured 48 hours, using the trapezoidal rule), Tmax (time to observe maximum drug concentration), K_{el} (apparent first order terminal rate constant calculated from a semi-log plot of the blood concentration versus time curve, using the method of least square regression) and $t_{1/2}$ (terminal half-life as determined by quotient 0.693/Kel).

All the samples were analyzed by the developed method and the mean concentrations vs time profile of Gemcitabine is shown in Figure-5. The pharmacokinetic parameters estimated are shown in Table 11.

Incurred sample reanalysis (ISR)

Re-analysis of all the dried VAMS, wet VAMS and blood–water (1:1, v/v) study sample sets demonstrated satisfactory ISR results between the original and the repeat result being within 20% of the mean of the two values. The lower agreement rate for the dried VAMS compared to the other two groups probably reflects the fact that the original and repeat dried samples were derived from physically separate sampling events with the VAMS device. Actually, the assay original and repeat analyses for the wet VAMS and blood–water samples were derived from the same liquid pool after the addition of water.

CONCLUSIONS

Apart from the UPLC method validation, it has also been demonstrated that the changes in assay bias and analyte recovery with HCT are acceptable with VAMS device. It was also demonstrated that temperature of the blood did not affect the assay result obtained. Thus, VAMS tips can be filled from

blood straight from the rat tail with a suitable blood draw technique, without having to wait for it to equilibrate to an ambient temperature. One of the rationales for adopting a microsampling approach is to reduce the amount of blood drawn at each sampling time point, which includes not just the blood collected for the analysis, but also any spilt blood and losses that occurs during staunching of the wound. Although the VAMS tips were overwhelmed, there is good agreement between the replicate VAMS samples for both dry and wet samples, taken at the same time point. The concentrations between the original and replicate results obtained for the dry VAMS samples showed 10% of the samples having a difference greater than 20%. This comparison complies with the ISR criteria and indicating that the volume of blood collected on the tip at any one time point was consistent. Thus the VAMS technique has the ability to replace DBS for quantitative bioanalysis, since it retains all the recognized advantages of DBS as well as making the sample collection process simpler, and reduce the work flow within the bioanalytical laboratory and minimizes the effect of HCT on assay bias.

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Conflict of Interest

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. All authors read and approved the final manuscript.

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Table 1: Linearity data of Gemcitabine

Concn (ng/mL)	Peak Area
50	1835
100	3525
150	5355
200	7368
250	9340
300	10951
400	14402
500	19309
y = 38.141x - 286.23 R^2 = 0.997	

Table 2: Recovery Results of Gemcitabine

	LLOQ QC		LOW QC		MID QC		HIGH QC	
	50 ng/mL		150 ng/mL		250 ng/mL		400 ng/mL	
	Concn found	% Recovery	Concn found	% Recovery	Concn found	% Recovery	Concn found	% Recovery
Recovery	56.204	112.407	156.722	104.466	250.114	99.927	394.569	98.642
	49.908	99.816	149.448	99.617	251.196	100.359	400.841	100.210
	55.226	110.451	140.671	93.767	253.271	101.188	393.941	98.485
	50.601	101.202	162.704	108.454	250.863	100.226	396.766	99.191
	55.107	110.214	156.716	104.462	253.486	101.274	393.556	98.389
	52.204	104.408	142.322	94.868	256.993	102.675	408.985	102.246
N	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
Mean	**53.208**	**106.416**	**151.431**	**100.939**	**252.654**	**100.942**	**398.110**	**99.527**
SD	**2.659**		**8.783**		**2.517**		**5.970**	
CV(%)	**4.997**		**5.800**		**0.996**		**1.499**	

Table 3: Intra-day Precision & Accuracy Results

Gemcitabine								
	LLOQ QC		LOW QC		MID QC		HIGH QC	
	50 ng/mL		150 ng/mL		250 ng/mL		400 ng/mL	
	Concn found	% Recovery	Concn found	% Recovery	Concn found	% Recovery	Concn found	% Recovery
Intra-day	52.914	105.827	149.646	99.618	244.790	97.684	387.033	96.758
	54.313	108.626	140.639	93.622	251.236	100.257	397.367	99.342
	51.447	102.894	143.223	95.342	246.306	98.289	395.875	98.969
	54.345	108.690	144.673	96.307	250.196	99.842	403.859	100.965
	52.886	105.772	145.144	96.621	255.884	102.111	384.929	96.232
	52.779	105.559	148.890	99.114	252.780	100.873	397.899	99.475
N	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
Mean	**53.114**	**106.228**	**145.369**	**96.771**	**250.199**	**99.843**	**394.494**	**98.623**
SD	**1.090**		**3.412**		**4.110**		**7.164**	
CV(%)	**2.051**		**2.347**		**1.643**		**1.816**	

Table 4: Inter-day Precision & Accuracy Results

Gemcitabine								
	LLOQ QC		LOW QC		MID QC		HIGH QC	
	50 ng/mL		150 ng/mL		250 ng/mL		400 ng/mL	
	Concn found	% Recovery	Concn found	% Recovery	Concn found	% Recovery	Concn found	% Recovery
Inter-day	55.496	110.991	152.974	101.968	252.305	100.803	410.627	101.922
	55.473	110.947	141.284	94.176	254.237	101.574	404.305	100.353
	55.771	111.542	154.625	103.068	252.745	100.978	402.002	99.781
	55.193	110.386	148.136	98.743	251.368	100.428	406.329	100.855
	55.020	110.039	160.512	106.992	251.450	100.461	399.015	99.039
	49.231	98.462	145.385	96.909	256.470	102.466	413.311	102.588
N	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
Mean	**54.364**	**108.728**	**150.486**	**100.309**	**253.096**	**101.118**	**405.931**	**100.756**
SD	**2.528**		**6.929**		**1.956**		**5.340**	
CV(%)	**4.650**		**4.604**		**0.773**		**1.316**	

Table 5: Long term stability study Results (n-6) after 60 days

	Gemcitabine			
	0 Hr-Low QC	0 Hr-HQC	Day-60-LQC	Day-60-HQC
	Conc found	Conc found	Conc found	Conc found
Long term stability after 60 days	157.217	386.091	152.355	385.579
	150.549	404.247	153.690	399.456
	151.185	408.218	151.050	411.678
	149.622	392.919	147.750	381.747
	142.374	406.491	145.350	389.795
	152.960	419.751	146.670	417.106
N	**6**	**6**	**6**	**6**
Mean	**150.651**	**402.953**	**149.478**	**397.560**
SD	**4.864**	**11.909**	**3.359**	**14.414**
CV(%)	**3.229**	**2.955**	**2.247**	**3.626**
% Change	**n/a**	**n/a**	**-0.779**	**-1.338**

Table 6: Short term stability study Results (n-6) for LOW QC concentration

	Gemcitabine					
	LOW QC					
	150 ng/mL					
	0 Hour		4 Hour		24 Hour	
	Conc found	% Recovery	Conc found	% Recovery	Conc found	% Recovery
Short term stability	157.217	104.812	152.550	101.700	155.880	103.920
	150.549	100.366	153.015	102.010	149.910	99.940
	151.185	100.790	147.180	98.120	155.811	103.874
	149.622	99.748	144.834	96.556	158.746	105.830
	142.374	94.916	143.442	95.628	139.535	93.023
	152.960	101.973	145.594	97.062	155.769	103.846
N	**6**	**6**	**6**	**6**	**6**	**6**
Mean	**150.651**	**100.434**	**147.769**	**98.513**	**152.608**	**101.739**
SD	**4.864**		**4.069**		**7.026**	
CV(%)	**3.229**		**2.754**		**4.604**	
% Change	**n/a**		**-1.913**		**1.299**	

Table 7: Short term stability study Results (n-6) for High QC concentration

	Gemcitabine					
	High QC					
	400 ng/mL					
	0 Hour		4 Hour		24 Hour	
Short term stability	Conc found	% Recovery	Conc found	% Recovery	Conc found	% Recovery
	386.091	96.523	392.287	98.072	381.136	95.284
	404.247	101.062	400.885	100.221	394.437	98.609
	408.218	102.055	411.518	102.879	400.426	100.106
	392.919	98.230	395.315	98.829	388.471	97.118
	406.491	101.623	412.094	103.023	409.955	102.489
	419.751	104.938	413.865	103.466	406.721	101.680
N	**6**	**6**	**6**	**6**	**6**	**6**
Mean	**402.953**	**100.738**	**404.327**	**101.082**	**396.858**	**99.214**
SD	**11.909**		**9.392**		**11.000**	
CV(%)	**2.955**		**2.323**		**2.772**	
% Change	**n/a**		**0.341**		**-1.513**	

Table 8: Freeze thaw stability (after III cycle) study Results (n-6) conducted below -20°C

	Gemcitabine			
	Freeze Thaw Cycle-III below -20°C			
	LOW QC		HIGH QC	
	150 ng/mL		400 ng/mL	
Freeze Thaw Cycle-III	Conc found	% Recovery	Conc found	% Recovery
	143.670	95.780	391.251	97.813
	148.890	99.260	384.471	96.118
	145.542	97.028	398.997	99.749
	143.367	95.578	396.982	99.245
	157.107	104.738	393.286	98.322
	159.630	106.420	400.974	100.243
N	**6**	**6**	**6**	**6**
Mean	**149.701**	**99.801**	**394.327**	**98.582**
SD	**7.041**		**6.012**	
CV(%)	**4.703**		**1.525**	

Table 9: Freeze thaw stability (after III cycle) study Results (n-6) conducted below -50°C

	Gemcitabine			
	Freeze Thaw Cycle-III below -50°C			
	LOW QC		HIGH QC	
	150 ng/mL		400 ng/mL	
Freeze Thaw Cycle-III	Conc found	% Recovery	Conc found	% Recovery
	143.130	95.420	408.431	102.108
	147.390	98.260	399.229	99.807
	150.600	100.400	387.387	96.847
	147.000	98.000	386.616	96.654
	138.390	92.260	387.888	96.972
	144.510	96.340	411.111	102.778
N	**6**	**6**	**6**	**6**
Mean	**145.170**	**96.780**	**396.777**	**99.194**
SD	**4.203**		**11.115**	
CV(%)	**2.895**		**2.801**	

Table 10: Matrix effect Results

Unit No.	Gemcitabine	
	250 ng/mL	
	Neat standard sample Concentration	Extracted blank plus spiked sample peak concentration
Unit No.: 1	9818	9040
Unit No.: 2	9194	8960
Unit No.:3	9391	9010
Unit No.: 4	9038	8828
Unit No.: 5	9789	9677
Unit No.: 6	9482	9274
N	**6**	**6**
Mean	**9452.000**	**9131.500**
SD	**313.083**	**304.150**
CV(%)	**3.312**	**3.331**
Matrix effect (%)	**0.966**	

Table. 11: Pharmacokinetic parameters of Gemcitabine in rat blood (n=6, Mean ± SD)

Parameter	Gemcitabine
C_{max} (ng/mL)	202.653 ± 20.551
T_{max} (h)	0.5 ± 0.025
$t_{1/2}$ (h)	0.5 ± 0.222
K_{el} (h^{-1})	0.0693 ± 0.046

Cmax: maximum blood concentration.

Tmax: time point of maximum blood concentration.

$t_{1/2}$: half life of drug elimination during the terminal phase.

K_{el}: elimination rate constant

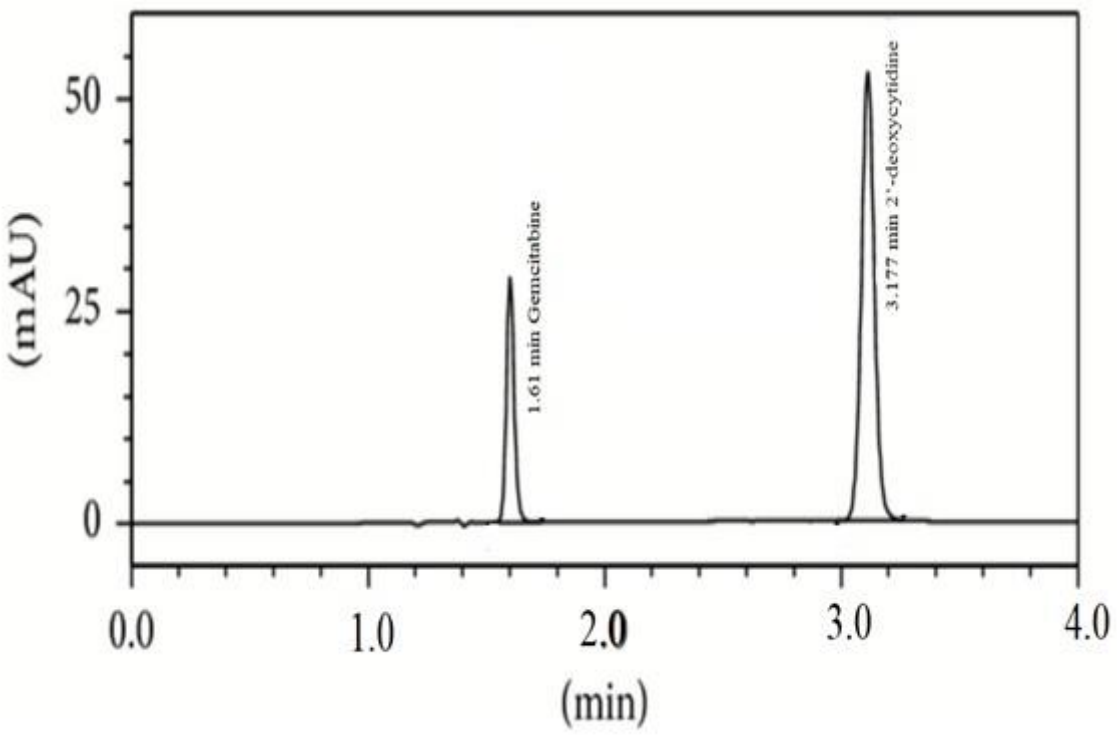

Figure 1: LLOQ chromatogram showing the separation of the analyte from IS

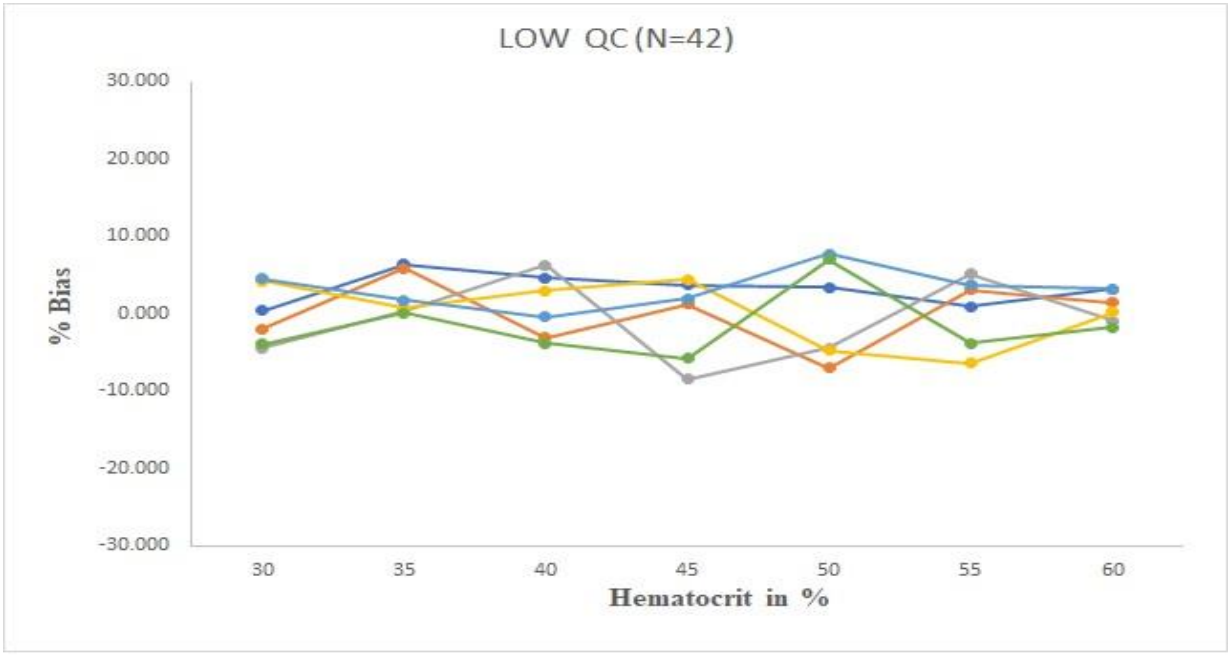

Figure 2: Influence of hematocrit on Low QC samples

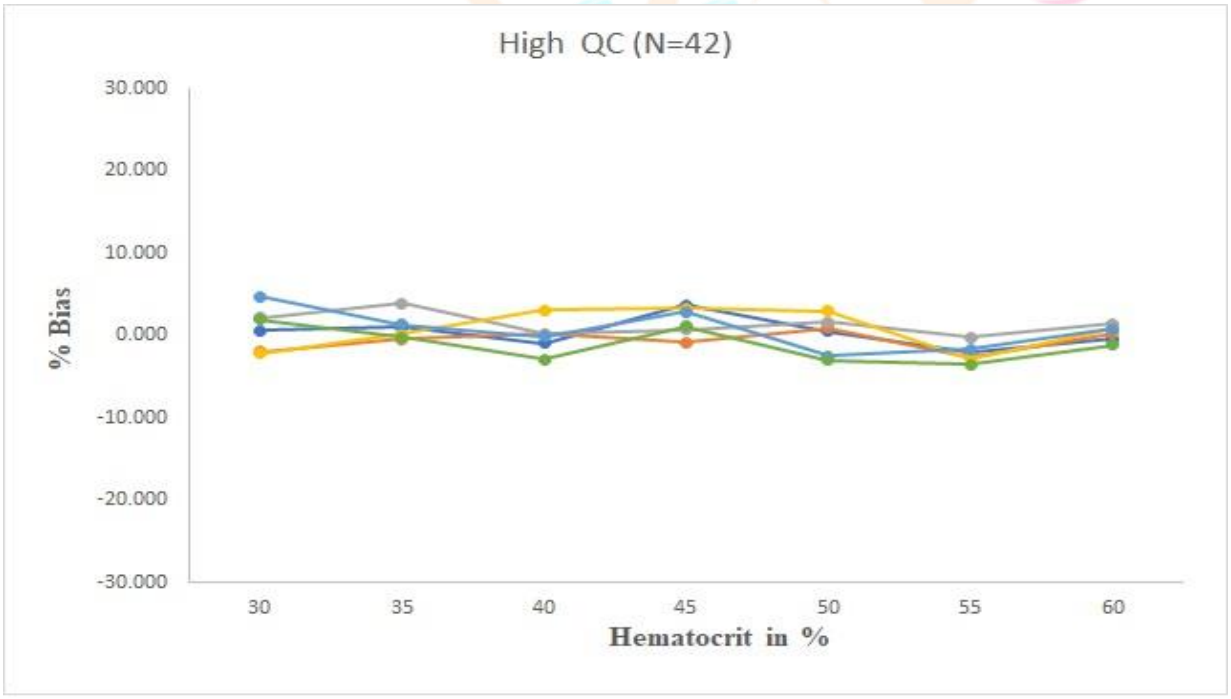

Figure 3: Influence of hematocrit on High QC samples

Figure 4: PK study and sample collection by VAMS sampler

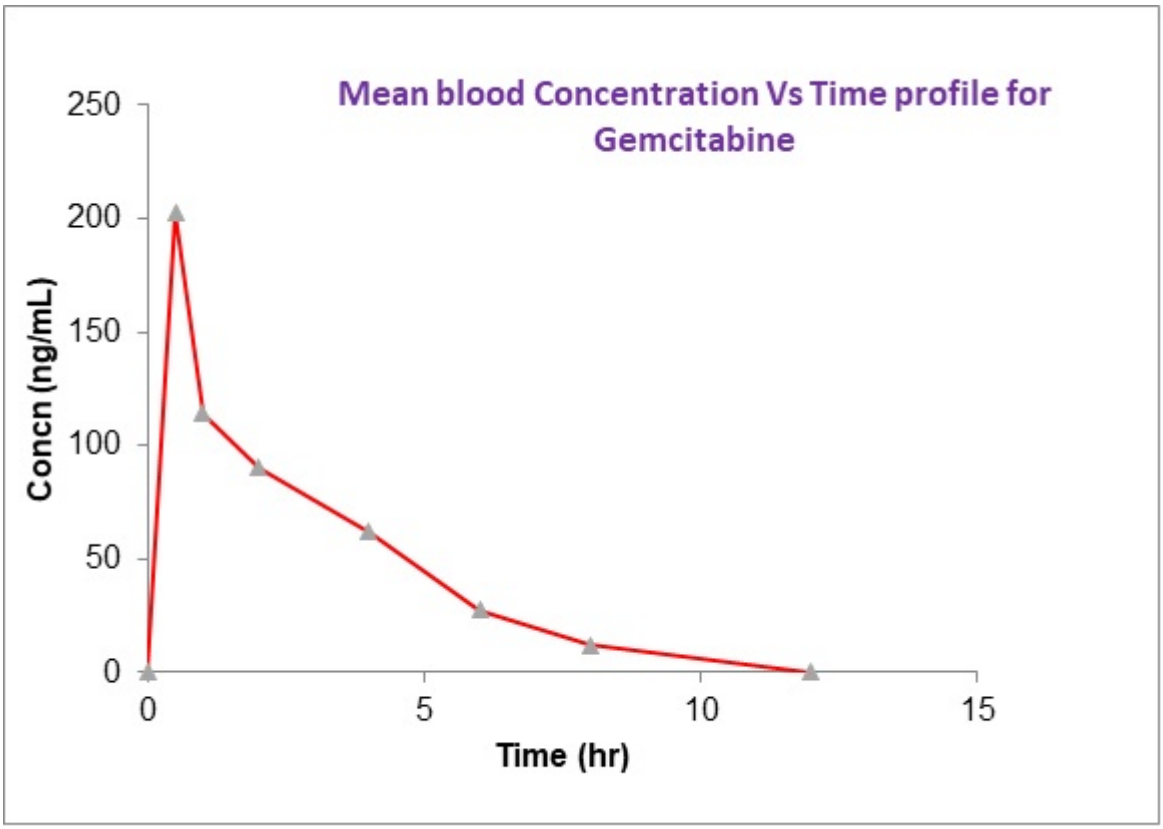

Figure 5: Mean blood concn-time profile curve of Gemcitabine in rats