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UPLC Method Validation For Bioanalysis Of Gilteritinib In Rat PK Study Using VAMS Methodology (Volumetric Absorptive Microsampling)

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ABSTRACT

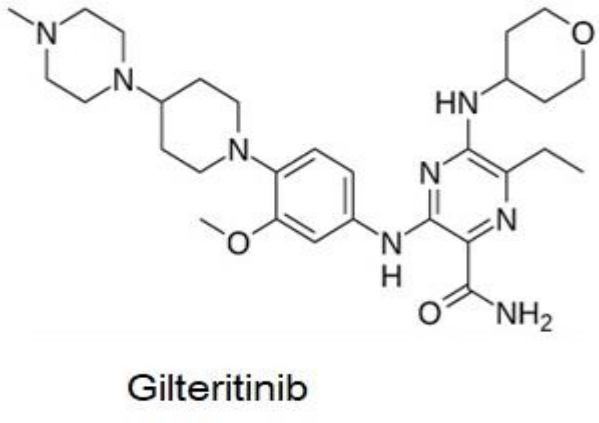
Volumetric absorptive microsampling (VAMS) is a simple intuitive technique for collecting and quantitative analysis of dried blood samples. It enables the collection of an accurate blood volume regardless of blood hematocrit. A bioanalytical method for the determination of Gilteritinib in dried blood 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-formic acid pH 3.5 (40-60) as the mobile phase at a flow rate of 0.8 mL/min. Gilteritinib 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 Gilteritinib were 93.2–109.2% and 93.7–115.1% respectively, and the precision (RSD, %) was less than 15% for both intra-day and inter-day measurements. Gilteritinib has a good linear relationship in the range of 10-1000 ng/mL with r2 value of 0.991. A robust and reliable UPLC method was fully optimized and developed to detect the blood concentration of Gilteritinib in rats and the samples were analyzed by Empower software.

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

INTRODUCTION

Gilteritinib, a novel, potent anti-cancer drug, is recently approved in Japan and USA for the treatment of adult patients who have relapsed or refractory acute myeloid leukemia (AML) with a FLT3 mutation[1]. It acts as an inhibitor of FLT3, hence it is a tyrosine kinase inhibitor[2]. **Gilteritinib**, sold under the brand name **Xospata (40 mg Tab)**. Gilteritinib was developed by Astellas Pharma and FDA approved on November 28, 2018. This drug was approved after being designed as an orphan drug with a fast track and priority review status.

Gilteritinib is a potent selective inhibitor of both of the mutations, internal tandem duplication (ITD) and tyrosine kinase domain (TKD), of the FLT3 receptor. In the same note, gilteritinib also inhibits AXL and ALK tyrosine kinases. FLT3 and AXL are molecules involved in the growth of cancer cells. The activity of gilteritinib permits an inhibition of the phosphorylation of FLT3. Gilteritinib (Figure 1) is primarily metabolized in the liver by the activity of CYP3A4. Its metabolism is driven by reactions of N-dealkylation and oxidation which forms the metabolite M17, M16 and M10. From the plasma concentration, the major form is the unchanged drug[3].



Gilteritinib

Figure 1: Chemical structure of Gilteritinib

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 Gilteritinib in rat blood by Volumetric Absorptive microsampling (VAMS)[4] 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[5]. 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[6,7]. Further, the design of the sampling device readily enables automation using standard liquid handling robots.

Very few chromatographic methods are published for Gilteritinib by LC-MS/MS in rat plasma[8], mouse plasma[9] and rat plasma[10]. Only one HPLC[11] method has been published for bioanalysis of this drug. Till date no UPLC method is reported for Gilteritinib in any matrix.

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 Gilteritinib concentrations in rat 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 formic acid pH 3.5 (40:60) in isocratic mode pumped at a flow rate of 0.8 mL/min. Analysis was performed for 3 min at the detection wavelength of 250 nm and the injection volume was 10 µL. The autosampler maintained at 4°C

Chemicals

Gilteritinib and internal standard (Neratinib) 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). Formic acid (GR grade) was purchased from Merck Chemicals Ltd., Mumbai. Water used in the entire analysis was prepared from Milli-Q water purification system from Millipore (Milford, MA, USA). Biological matrix (rat 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 Gilteritinib was prepared by dissolving standard 50 mg of Gilteritinib 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 rat 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 rat blood to yield final concentrations of calibration samples 10, 50, 100, 250, 500, 600, 800 and 1000 ng/mL. The final concentration of IS was 100 ng/mL. Similarly, QC samples were prepared at four concentration levels LLOQ (10 ng/mL), LQC (100 ng/mL) MQC (500 ng/mL) and HQC (800 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 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 rats. 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 rats 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 acetonitrile 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 gilteritinib 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 lysis to occur. The wet VAMS blood–water samples were either used immediately, or

stored frozen at -20°C. Gilteritinib was extracted from aliquots of the wet VAMS blood–water samples by protein precipitation, following the addition of 5 volumes of acetonitrile 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 acetonitrile 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[12] and the ICH guidelines[13] 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 10 to 1000 ng/mL gilteritinib 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 gilteritinib/IS versus the concentration of gilteritinib. Gilteritinib 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, 800 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 gilteritinib 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 gilteritinib 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 gilteritinib in extracted validation samples versus blank samples spiked after extraction. The absolute matrix effect was calculated from the ratio of mean peak areas of gilteritinib 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 gilteritinib and IS. No more than 10% of the blank samples could have peak areas greater than 20% of the average peak area of gilteritinib 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). Gilteritinib 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 formic acid pH 3.5 (40:60) as mobile phase. Variation of the column temperature between 20°C 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 0.8 mL/min. The method allowed the separation of analyte with IS in 3 min (Figure 2) 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 10 to 1000 ng/mL

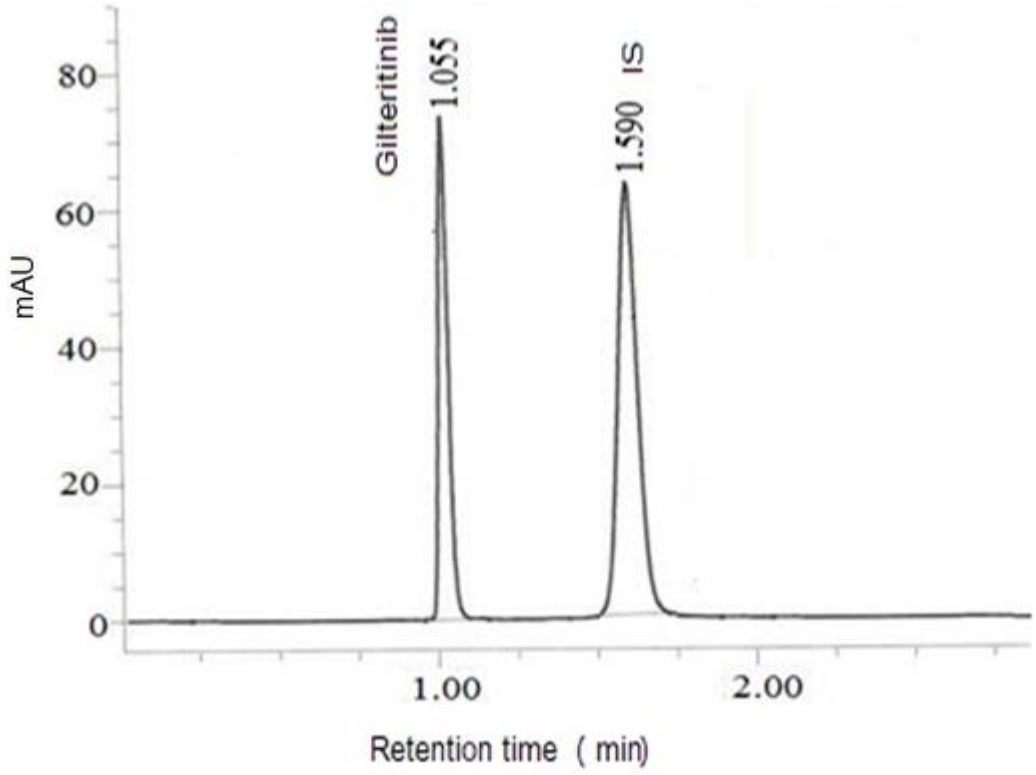


Figure 2: LLOQ chromatogram showing the separation of the analyte from IS

Table 1: Linearity data of Gilteritinib

Concn (ng/mL)	Peak Area
10	40652
50	91425
100	180652
200	321425
500	606977
600	677785
800	853069
1000	1029005
y = 985.52x + 73524	R^2 = 0.991

The limit of detection by UPLC was found to be 2 ng/mL and LOQ was found to be 10 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 rat blood were in the range of 93.2–115.1% with relative standard deviations less than 10.0%, which indicates the sample preparation technique is suitable for extracting (Table 2).

Table 2: Recovery Results of Gilteritinib

	LLOQ QC		LOW QC		MID QC		HIGH QC	
	10 ng/mL		100 ng/mL		500 ng/mL		800 ng/mL	
	Concn found	% Recovery	Concn found	% Recovery	Concn found	% Recovery	Concn found	% Recovery
Recovery	11.099	109.351	101.983	101.938	512.305	102.340	814.627	101.462
	11.095	109.307	94.189	94.148	514.237	102.726	800.305	99.679
	11.154	109.893	103.083	103.038	510.745	102.028	808.002	100.637
	11.039	108.754	98.757	98.714	514.368	102.752	826.329	102.920
	11.004	108.413	107.008	106.961	500.450	99.971	790.015	98.397
	9.846	97.006	96.923	96.880	513.470	102.572	833.311	103.790
N	**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
Mean	**10.873**	**107.121**	**100.324**	**100.280**	**510.929**	**102.065**	**812.098**	**101.148**
SD	**0.506**		**4.619**		**5.310**		**16.140**	
CV(%)	**4.650**		**4.604**		**1.039**		**1.987**	

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 93.2–109.2% and inter-day accuracies were in the range of 93.7–115.1%. The recovery results are displayed in Table 3 and Table 4.

Table 3: Intra-day Precision & Accuracy Results

Gilteritinib								
	LLOQ QC		LOW QC		MID QC		HIGH QC	
	10 ng/mL		100 ng/mL		500 ng/mL		800 ng/mL	
Intra-day	Concn found	% Recovery	Concn found	% Recovery	Concn found	% Recovery	Concn found	% Recovery
	10.858	106.978	104.145	103.689	499.572	99.796	797.033	99.629
	10.863	107.020	101.260	100.816	504.472	100.775	767.367	95.921
	10.289	101.373	103.121	102.669	499.613	99.804	791.875	98.984
	11.087	109.231	93.216	92.808	502.393	100.360	833.859	104.232
	11.058	108.943	104.504	104.046	513.768	102.632	764.929	95.616
	10.956	107.937	99.112	98.678	502.560	100.393	767.899	95.987
Mean	**10.852**	**106.914**	**100.893**	**100.451**	**503.730**	**100.627**	**787.160**	**98.395**
SD	**0.292**		**4.262**		**5.267**		**26.674**	
CV(%)	**2.686**		**4.224**		**1.046**		**3.389**	

Table 4: Inter-day Precision & Accuracy Results

Gilteritinib								
	LLOQ QC		LOW QC		MID QC		HIGH QC	
	10 ng/mL		100 ng/mL		500 ng/mL		800 ng/mL	
	Concn found	% Recovery	Concn found	% Recovery	Concn found	% Recovery	Concn found	% Recovery
Inter-day	11.224	110.582	110.448	110.400	507.114	101.303	774.957	96.870
	9.816	96.708	99.632	99.588	513.196	102.518	803.841	100.480
	11.689	115.163	93.781	93.740	505.271	100.934	783.941	97.993
	10.202	100.516	108.470	108.422	503.863	100.653	790.766	98.846
	10.214	100.629	104.478	104.432	513.486	102.576	783.556	97.945
	10.744	105.853	94.882	94.840	513.993	102.677	807.985	100.998
Mean	**10.648**	**104.909**	**101.948**	**101.903**	**509.487**	**101.777**	**790.841**	**98.855**
SD	**0.708**		**6.974**		**4.584**		**12.774**	
CV(%)	**6.646**		**6.841**		**0.900**		**1.615**	

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 Gilteritinib 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.

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

	Gilteritinib			
	0 Hr-LQC	**0 Hr-HQC**	**Day-60-LQC**	**Day-60-HQC**
	Conc found	**Conc found**	**Conc found**	**Conc found**
Long term stability after 60 days	99.585	802.369	95.624	797.831
	92.581	820.572	91.258	785.801
	96.321	814.652	97.585	817.382
	98.548	799.542	99.633	795.932
	88.548	789.484	90.258	803.011
	91.259	788.200	92.370	786.414
Mean	**94.474**	**802.470**	**94.455**	**797.729**
SD	**4.363**	**13.091**	**3.746**	**11.728**
CV(%)	**4.618**	**1.631**	**3.965**	**1.470**
% Change	**n/a**	**n/a**	**-0.0202**	**-0.59084**

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.

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

	Gilteritinib					
	LOW QC 100 ng/mL					
	0 Hour		4 Hour		24 Hour	
	Conc found	**% Recovery**	**Conc found**	**% Recovery**	**Conc found**	**% Recovery**
Short term stability	97.019	97.019	100.183	100.183	99.123	99.123
	95.199	95.199	95.586	95.586	94.579	94.579
	102.939	102.939	101.670	101.670	99.346	99.346
	97.998	97.998	96.566	96.566	96.620	96.620
	105.215	105.215	104.691	104.691	102.338	102.338
	95.946	95.946	96.640	96.640	96.551	96.551
Mean	**99.053**	**99.053**	**99.223**	**99.223**	**98.093**	**98.093**
SD	**4.070**		**3.571**		**2.740**	
CV(%)	**4.109**		**3.599**		**2.794**	
% Change	**n/a**		**0.17161**		**-0.9691**	

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

	Gilteritinib					
	High QC 800 ng/mL					
	0 Hour		4 Hour		24 Hour	
	Conc found	% Recovery	Conc found	% Recovery	Conc found	% Recovery
Short term stability	811.956	101.494	790.037	98.755	792.187	99.023
	832.389	104.049	789.922	98.740	784.419	98.052
	810.579	101.322	809.993	101.249	804.251	100.531
	795.544	99.443	810.124	101.266	809.152	101.144
	785.537	98.192	770.028	96.254	776.907	97.113
	784.259	98.032	798.124	99.766	784.419	98.052
Mean	**803.377**	**100.422**	**794.705**	**99.338**	**791.889**	**98.986**
SD	**18.506**		**15.078**		**12.546**	
CV(%)	**2.303**		**1.897**		**1.584**	
% Change	**n/a**		**-1.07952**		**-1.42999**	

Gilteritinib was stable upto 10 h on bench top at room temperature and over 4 freeze–thaw cycles. In rat blood, the freeze-thaw study was carried out and the results are presented in Table 8 and Table 9.

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

	Gilteritinib			
	Freeze Thaw Cycle-IV below -20°C			
	LOW QC		HIGH QC	
	100 ng/mL		800 ng/mL	
	Conc found	% Recovery	Conc found	% Recovery
Freeze Thaw Cycle-IV	102.298	102.298	808.830	101.104
	103.139	103.139	797.516	99.690
	101.581	101.581	806.918	100.865
	100.358	100.358	813.996	101.750
	99.005	99.005	799.341	99.918
	101.042	101.042	801.437	100.180
Mean	**101.237**	**101.237**	**804.673**	**100.584**
SD	**1.460**		**6.314**	
CV(%)	**1.442**		**0.785**	

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

	Gilteritinib			
	Freeze Thaw Cycle-IV below -50°C			
	LOW QC		HIGH QC	
	100 ng/mL		800 ng/mL	
Freeze Thaw Cycle-IV	Conc found	% Recovery	Conc found	% Recovery
	90.156	90.156	797.831	99.729
	99.300	99.300	785.115	98.139
	97.704	97.704	797.147	99.643
	94.309	94.309	795.105	99.388
	98.517	98.517	803.587	100.448
	100.430	100.430	806.414	100.802
Mean	**96.736**	**96.736**	**797.533**	**99.692**
SD	**3.834**	**3.834**	**7.428**	**0.928**
CV(%)	**3.964**	**3.964**	**0.931**	**0.931**

The variability of the matrix effect in rat 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.

Table 10: Matrix effect Results

Unit No.	Gilteritinib	
	500 ng/mL	
	Neat standard sample Concentration	Extracted blank plus spiked sample peak concentration
Unit No.: 1	48818	48040
Unit No.: 2	48194	44560
Unit No.:3	48391	46510
Unit No.: 4	49038	46287
Unit No.: 5	47789	44677
Unit No.: 6	48332	46274
N	**6**	**6**
Mean	**48427.000**	**46058.000**
SD	**446.734**	**1294.827**
CV(%)	**0.922**	**2.811**
Matrix effect (%)	**0.951**	

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 gilteritinib 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 3) and high QC level (Figure 4) and proved to be promising over an acceptable range.

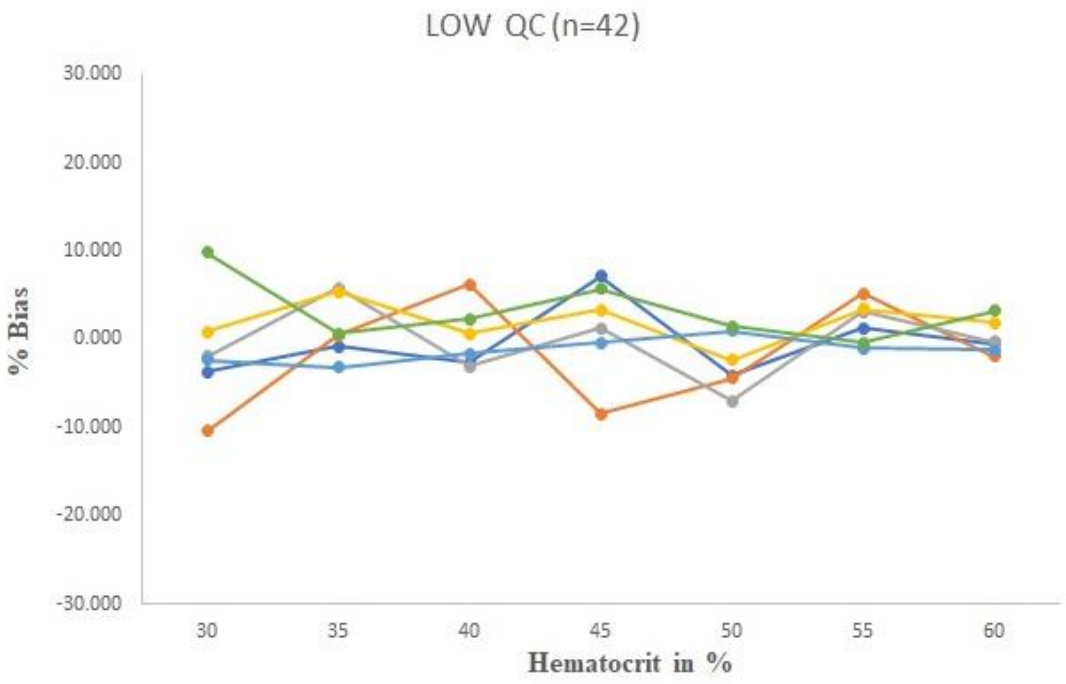


Figure 3: Influence of hematocrit on Low QC samples

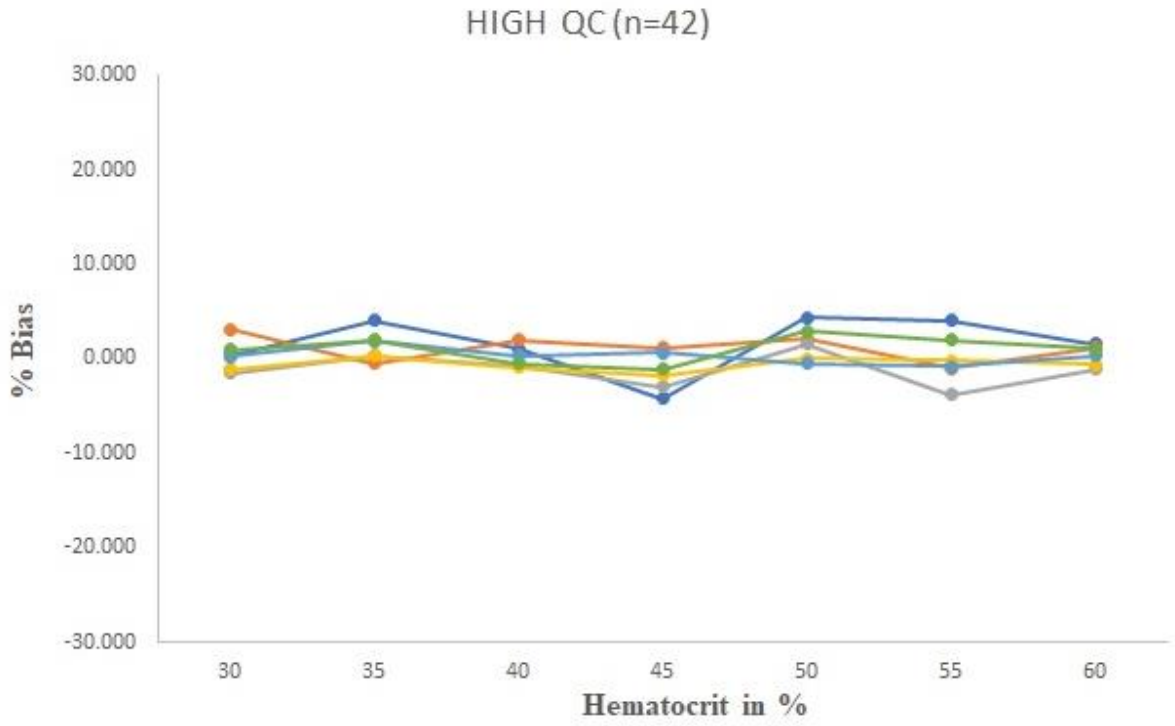


Figure 4: Influence of hematocrit on High QC samples

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 gilteritinib 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 5). 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 acetonitrile 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).

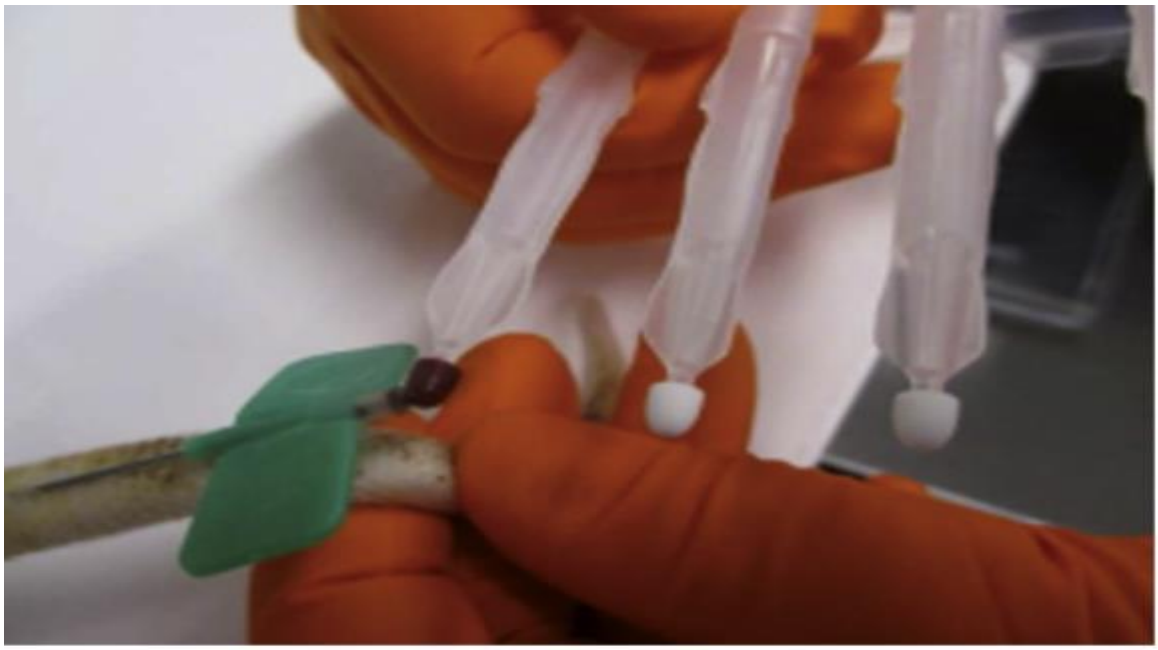


Figure 5: Sample collection by VAMS sampler

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 gilteritinib is shown in Figure 6. The pharmacokinetic parameters estimated are shown in Table 11.

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

Parameter	Gilteritinib
C_{max} (ng/mL)	227.947 ± 59.399
T_{max} (h)	4.0 ± 2.0
$t_{1/2}$ (h)	45 ± 5.2
K_{el} (h^{-1})	0.0154 ± 0.00148

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

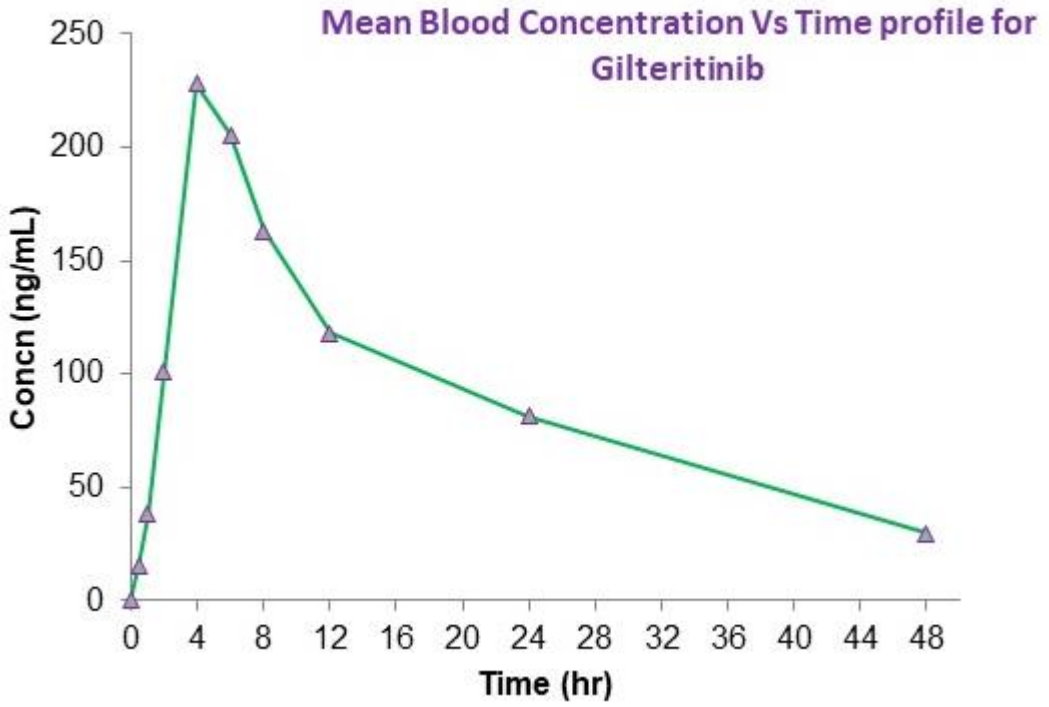


Figure 6: Mean blood concn-time profile curve of Gilteritinib in rats

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.

Statements and Declarations

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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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