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Original Article

DEVELOPMENT AND VALIDATION OF A DRIED BLOOD SPOT LC-MS/MS ASSAY TO QUANTIFY GEMCITABINE IN HUMAN WHOLE BLOOD: A COMPARISION WITH AND WITHOUT CYTIDINE DEAMINASE INHIBITOR

SUJAN KUMAR DP[1], PALAVAN C[1], SESHAGIRI RAO JVLN[2]*

[1]College of Pharmaceutical Sciences, Andhra University, Visakhapatnam 530003, Andhra Pradesh, India, [2]Srinivasa rao College of Pharmacy, Madhurawada, Visakhapatnam 530041, India

Email: sujanmpharm@gmail.com

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ABSTRACT

Objective: The purpose of this paper is to develop and validate a LC-MS/MS method for the quantification of gemcitabine in whole human blood using dried blood spots**.**

Methods: Gemcitabine fortified blood samples without tetrahydrouridine were spotted (50 µl) onto the DBS cards and dried for 2h at ambient room temperature. 3 mm punched spots were extracted by acetonitrile: water (90:10v/v) containing carbamazepine as internal standard (IS). Analyte and IS were separated on BDS Hypersil C18,(100 X 4.6 mm, 5 µ) column using a mixture of methanol and 2 mM ammonium acetate buffer (65:35 v/v) at a flow rate of 0.5 mL/min. Detection involved API-4000 LC-MS/MS with electrospray ionization in the positive ion mode.

Results: The assay was validated over the concentration range of 5-5000 ng/ml. Intra and inters assay precision values (% CV) were less than 6.0% while the accuracy was within±15%. The mean recovery (%CV) of gemcitabine from DBS was ≥83.5% (≤4.0). Hematocrit values ranging between 0.25 and 0.62 were within acceptable limit with accuracy 93.0-103.1% of nominal values and %CV of ≤ 6.5 across the LQC and HQC levels. Gemcitabine was stable on DBS cards for atleast 90days at room temperature.

Conclusions: A cytidine drug like gemcitabine exhibits *ex vivo* instability and rapidly converts to inactive metabolite in blood. All current published methods for stabilisation include tetrahydrouridine a cytidine deaminase inhibitor in the sample collection tubes. The proposed DBS method can be used as an alternative assay to conventional plasma analysis without adding enzyme inhibitor.

Keywords: Gemcitabine, dFdc, LC-MS/MS, Dried blood spot, Tetrahydrouridine.

INTRODUCTION

Pancreas cancer is the fourth commonest cause of cancer-related mortality across the world, with incidence equalling mortality [1]. Untreated metastatic pancreatic cancer has a median survival of 3–5 months and 6–10 months for locally advanced disease [2]. The majority of cases are diagnosed in the advanced stages, making curative therapy impossible and leading to poor prognosis [3]. Gemcitabine (2, 2-difluoro-2-deoxycytidine, dFdC) is a novel deoxycytidine analog [4] administered as the intravenous infusion to treat human malignancies including pancreatic [5], ovarian [6], breast [7] and bladder [8]. By deoxycytidine kinase, gemcitabine phosphorylates intracellularly to an active metabolites 2, 2-diXuorodeoxycytidine-5_-diphosphate (dFdCDP) and triphosphate (dFdCTP) inhibiting the processes required for DNA synthesis. Incorporation of dFdCTP into DNA is most likely the major mechanism by which gemcitabine causes cell death. [9] Gemcitabine is metabolically inactivated by cytidine deaminase (CDA) into the metabolite 2, 2-diXuorodeoxyuridine (dFdU) [10]. Several analytical methods have been proposed for the measurement of gemcitabine in plasma/serum or tissues. Most commonly used methods were HPLC-UV [11-17] with a run time of more than 20 mins and require a minimum plasma volume of 200 µl with a detection limit range between 0.1 to 2.6µg/ml. HPLC coupled to mass spectrometric detection offers a wide range of advantages in support of bio analysis in terms of selectivity, sensitivity and faster analysis time. The published LC-MS/MS methods use a high volume of plasma [18, 19] or few had very long run time exceeding 15 min [20-22]. A method proposed by Bowen c, *et al.* [23] with a very short run time of 1.7 min involves a complicated derivatisation process of gemcitabine with dansyl chloride to retain it on the reverse phase column.

Dried blood spot (DBS) screening has been used as an alternative sample collection for pediatric purposes in 1960s [24]. DBS analysis provides several advantages, including reduced blood sample volumes collected for drug analysis and ease of collection, storage, and transportation [25, 26]. Additionally, DBS sample preparation, stability, and storage are usually most convenient and been successfully applied for analysing small molecules in different therapeutics areas like anti-malarials [27, 28] anti-retrovirals [29-31] and many more. Moreover, some compounds are unstable ex-vivo in biological fluids (e. g., blood or plasma) and require adapted procedures to prevent degradation [32, 33] in such cases; stability can be enhanced on DBS without the need for the aforementioned procedures [34, 35]. The objective of this method was to develop an alternative assay for stabilisation of gemcitabine avoiding addition of enzyme inhibitors and thus potentially minimising the errors in clinic in sample collection and also eliminating biohazard risks.

MATERIALS AND METHODS

Chemicals and materials

Gemcitabine hydrochloride reference sample (99.8% pure) was obtained from USP Reference Standard. Carbamazepine (99.24% pure) was used as an internal standard and was obtained from Clearsynth Labs (P) Ltd (Mumbai, India). Their chemical structures were shown in fig. 1. HPLC grade methanol and acetonitrile were purchased from JT Baker (Phillipsburg, USA). Analytical grade ammonium acetate was purchased from Merck Ltd (Mumbai, India). Tetrahydrouridine was purchased from Calbiochem (San Diego,USA). Water used for the LC–MS/MS analysis was prepared by using Milli Q water purification system procured from Millipore (Bangalore, India). FTA DMPK-A cards and Harris Uni-Core Punch, 3 mm were purchased from GE healthcare. The control human whole blood was procured from Deccan's Pathological Lab's (Hyderabad, India).

LC–MS/MS instrument and conditions

An HPLC system (Shimadzu, Kyoto, Japan) consisting of a binary LC–20AD prominence pump, an auto sampler (SIL–HTc) and a solvent

degasser (DGU–20A3) was used for the study. Aliquot of 10 µl of the extracted samples were injected onto the column, BDS Hypersil C18, (100 X 4.6 mm, 5 µ), which was kept at 40 ºC. An isocratic mobile phase consisting of a mixture of methanol and 2 mM ammonium acetate buffer (65:35 v/v) at a flow rate of 0.5 mL/min was used to separate the analyte from the endogenous components. Detection was carried out in the positive electospray ionisation mode for the analyte and the IS using an AB Sciex API–4000 mass spectrometer (Foster City, CA, USA) equipped with a Turboion spray ™ interface at 500 ºC. The ion spray voltage was set at 5500 V. The source parameters viz. the nebulizer gas (GS1), auxiliary gas (GS2), curtain gas and CAD were set at 45, 55, 20, and 6 psi, respectively. The compound parameters viz. declustering potential (DP) 45V for gemcitabine and 25V for carbamazepine, collision energy and collision cell exit potential were 25 V and 12v for both gemcitabine and carbamazepine. Detection of the ions was carried out in the multiple–reaction monitoring mode (MRM), by monitoring the transition pairs of *m/z* 264.2 precursor ion to the *m/z* 112.0 product ion for gemcitabine and *m/z* 237.3 precursor ion to the *m/z* 194.1 product ion for carbamazepine. Both Q1 and Q3 Quadrupoles were set on unit resolution. Data was processed by Analyst Software™ (version 1.6.1).

a) Gemcitabine **b) Carbamazepine**

Fig. 1: Chemical Structures of Gemcitabine and Carbamazepine

Preparation of stock solutions of the analyte and IS

Two different sets of primary stock solutions for gemcitabine were prepared by separate weighing, to get at a concentration of 1 mg/ml in water and were stored at 2-8 °C for their stability. Each set of primary stock solutions was suitably diluted with Water for the preparation of working standard solutions of calibration curve (CC) and quality control (QC) samples. A working IS dilution (4000 ng/ml) was prepared from the IS stock (methanol) 1 mg/ml in acetonitrile: water (90:10v/v).

Preparation of calibration curve standards and quality control samples

Calibration samples of gemcitabine were prepared at concentration levels of 5.0, 12.5, 25.0, 50.0, 125.0, 250.0, 1250.0, 2500.0, 4000.0 and 5000.0 ng/ml as a single batch at each concentration levels. Similarly, quality control (QC) samples were also prepared as a bulk based on an independent weighing of standard drug, at concentrations of 5.2 (LLOQ), 14.5 (low), 241.5 (middle 1), 1050.0 (middle 2) and 4200 ng/ml (high) as a single batch at each concentration. Both calibration standards and Quality control samples were prepared in human K2 EDTA blood. The final hematocrit in the prepared calibration standards and QC samples were about 0.435. A 50 µl aliquot of each calibration standard and QC sample was spotted onto the DBS cards. The cards were left on the bench at ambient room temperature for a minimum of 2h for complete drying of blood spots and then packed in a sealed plastic bag with small amount of desiccant.

Effect of tetrahydrouridine: plasma versus whole blood (dried blood spots)

Whole blood stability of gemcitabine in the presence and absence of cytidine deaminase inhibitor

In brief, the experiment was designed by spiking a known concentration at (HQC Level) separately into whole human blood in presence of THU (positive control) and in absence of THU (negative control). The spiked samples were left at room temperature and aliquots were removed at time points 0.00, 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8, and 24h from both control samples. Plasma was isolated by centrifugation at 1500g for 5 min and then frozen at-20ºc. The concentration of gemcitabine in plasma samples was determined as described below in sample extraction.

From the literature, recommended analytical methods [11-23] for the analysis of gemcitabine tetrahydrouridine a cytidine deaminase inhibitor must be added to the sample collection tubes prior to sample collection to prevent deamination of gemcitabine to inactive metabolite dFdU. A series of THU concentrations ranging from 1µg to 100µg was evaluated and a final concentration of 25µg/ml of THU was optimized in-house to inhibit the deamination activity.

Short term and long term stability of gemcitabine in presence and absence of THU on DBS cards

In parallel to the above experiment, a similar experiment was carried out by spiking gemcitabine at a concentration of LQC and HQC into pre-treated whole human blood with THU. 50 µl of the above was spotted onto the DBS cards (~60 spots were prepared at each level) and dried at room temperature for 2hr. An equal concentration of gemcitabine was spiked into whole human blood without THU at the same time. This was also spotted onto the fresh DBS cards (50 µl).

Sample extraction

A 3-mm disc was punched from each dried blood spot sample using Harris Uni-Core Punch and transferred to individual 1.5 ml eppendorf tubes. 200 µl of IS working solution (4000 ng/ml) was added to all samples, except matrix blanks to which 200 µl aliquot of acetonitrile/water (90/10, v/v) was added. The tubes were capped tightly and gently mixed for 20 minutes on Vortexer (Eppendorf mixmate) and then centrifuged at 14000g for 5 min. Finally 100 µl of each extract was transferred to HPLC vials and 10 µl aliquot of it was injected on to the column. The same procedure was followed for plasma samples by separating 50 µl of plasma from spiked whole human blood with gemcitabine to assess the stability studies in the presence and absence of THF.

Method validation

Although there is no specific regulatory guidelines exist for the validation of bioanalytical methods using DBS cards. The entire validation of the method was carried out as per FDA guidelines [36] (Bioanalytical Method Validation published in May 2001). The method was validated for various parameters like selectivity, sensitivity, matrix effect, linearity, precision, accuracy, recovery, dilution integrity, stability and also some additional experiments unique to DBS including spotting volume, and hematocrit were evaluated. Selectivity of the method was assessed by analyzing six different lots of blank human blood for interference at the retention time of gemcitabine and it's IS. The area responses of the interfering substances or noise at the retention times of the gemcitabine and IS were acceptable, if the % interference was less than 20% of the

mean response of the lowest standard in calibration curve point or LLOQ (n = 6). The area responses of the interfering substances or noise at the retention time of internal standard were acceptable, if the % interference was less than 5 % mean response of the internal standard areas in the 6 LLOQ samples.

Sensitivity was carried out by the noise or the response from six spiked LLOQ samples. The six replicates should have a precision of ≤ 20% and with an accuracy of±20% of the nominal value of the LLOQ. Matrix effect, expressed as IS normalized matrix factor (MF) was assessed by comparing the mean area response of post–extraction spiked samples with the mean area of aqueous samples (neat samples) prepared in mobile phase solutions at LQC and HQC levels. The overall precision of the matrix factor was expressed as coefficient of variation (CV).

$$\text{Matrix Factor} = \frac{\text{Peak response area ratio in presence of matrix ions}}{\text{Mean peak response area ratio in absence of matrix ions}}$$

Matrix effect was also evaluated with six different lots of human blood. Three replicate samples each of LQC and HQC were prepared from different lots of blood (36 QC samples in total).

The linearity of the method was determined for gemcitabine in the concentration range of 5-5000 ng/ml. As per the guidelines for the determination of linearity, a calibration curve should consist of a blank sample (matrix sample processed without analyte or internal standard), a zero sample (matrix sample processed without analyte but with internal standard), and at least six non-zero samples (matrix samples processed with analyte and internal standard) covering the expected range, including LLOQ.

The acceptance limit of accuracy for each of the back calculated concentration was±15% of their nominal values except LLOQ which it was±20% of the LLOQ concentration. For a calibration curve to be accepted at least 67% of nonzero standards should pass including the lower and the upper limit of quantification (LLOQ & ULOQ) concentration.

Intra day precision and accuracy were determined by analyzing six replicates at five different QC levels on the same day. Inter day precision and accuracy were determined by analyzing six replicates at five different QC levels four consecutive days The acceptance criteria for accuracy should within±15% standard deviation to their nominal concentrations except for LLOQ, it should be±20 and precision should within≤15% relative standard deviation (RSD) except for LLOQ, it should be ≤20%.

The recovery was calculated by analyzing extracted QC samples at three levels (LQC, MQC and HQC) with that of the aqueous equivalents, which were having the same concentration of the QC samples prepared in the mobile phase without any extraction procedure. Similarly, the recovery of the IS was determined by comparing the mean peak areas of the extracted QC samples for their IS area with the aqueous equivalent's IS area. The acceptance for the recovery was the RSD of the areas obtained for extracted and aqueous equivalents should be ≤15% individually at each level of QC samples and the RSD of the % recoveries for all of QC levels should be ≤ 15%. The recovery should be<115% at any level of concentration.

To demonstrate that a method is suitable for a DBS sample to quantify an analyte concentration higher than the ULOQ, the dilution integrity should be assessed. However, different from whole blood, plasma or serum which can be diluted using the same matrix blank prior to analysis a DBS itself cannot be diluted.

A simple approach is to dilute the extracted dilution QC sample using one or more of extracted DBS zero samples (containing IS only). The diluted QC sample was then analyzed along with the calibration standards. The obtained bias from the diluted QCs in six replicates should be within±15% of the nominal value and with %CV<15%.

Influence of hematocrit on assay quantitation was measured by preparing two different concentrations of gemcitabine at LQC and HQC level in whole human blood with varied hematocrit values (0.25 and 0.62) for both the QC levels respectively. A difference within±15% of the nominal values for the measured analyte concentrations from the hematocrit QC samples against a calibration standard that was prepared in fresh blood with a hematocrit value of 0.43% is acceptable showing a negligible hematocrit effect.

Dried blood spotting sample volume variability was evaluated by spotting volumes 20 µl and 30 µl at LQC and HQC levels. These QC samples were analysed against calibration standards prepared using 50 µl of blood volume. A difference within±15% of the nominal values would suggest no significant effect of blood volume on sensitivity.

Stability experiments were conducted to evaluate the gemcitabine stability at different storage and processing conditions. Auto sampler stability was evaluated by storing extracted QC samples in the auto sampler (4 °C) for 72 hours. Post preparative stability was evaluated to show that the integrity of the entire run is maintained, if reinjected after a specified number of hours.

This was performed by reinjecting an accepted analytical run, usually a precision and accuracy batch. Long-term stability of gemcitabine in human blood was established by analyzing DBS cards stored at room temperature for specific durations, The concentrations of the stored samples were determined using freshly made STDs and QCs. Samples were considered stable, if assay values were within the acceptance limits of accuracy should be±15% of their nominal concentrations and precision should be ≤15 RSD.

RESULTS

Mass spectrometry

Mass parameters were tuned in both "+" Ve and "–"Ve ionization modes. The more reproducible area was achieved in positive polarity mode for both analyte and IS. The data from MRM mode was considered to obtain better selectivity. Protonated $[M+H]^+$form of each analyte and IS was the parent ion in the Q1 spectrum. The same was used as the precursor ion to obtain Q3 production spectra. The most sensitive mass transitions were monitored from m/z 264.20 to 112 for gemcitabine and from 237.30 to 194.1 m/z for Carbamazepine.

Method development

Separation was attempted using various combinations of Methanol, acetonitrile and buffer with varying contents of each component on different columns; C_8 and C_{18} of different makes. The use of 2 mM ammonium acetate buffer (pH 6.4±0.1) was useful in achieving optimum reproducible response.

Mobile phase consisting of methanol and 2 mM ammonium acetate buffer (pH 6.4±0.1) was found to be suitable, at which the analytes were protonated and well separated. BDS Hypersil C18 (Thermo scientific; 100 mm X 4.6 mm, 5 µm) gave a good peak shape and response. The mobile phase drawn at a flow rate of 0.5 mL/min gave shorter run time of the chromatography. A simple mixture of acetonitrile: water (90:10v/v) as an extraction solvent proved to be rugged and provided the cleanest samples.

A suitable internal standard must mimic the analyte during extraction and compensate for any analyte on the column. After checking with several available compounds, finally carbamazepine was found to be the best to serve as an internal standard.

Selectivity

The selectivity of the method was examined by analyzing extracted dried blank human blood samples from six different sources. Typical chromatograms of extracted blank human blood spot (fig. 2A) and an extract spiked only with the IS (fig. 2B).

As shown in fig. 2A, no significant direct interference in the blank was observed from endogenous substances in drug–free human blood at the retention time of the analyte and the IS. Similarly, fig. 2B shows the absence of direct interference from the IS to the MRM channel of the analyte. Fig. 2C depicts a representative ion–chromatogram for the LLOQ sample (5.0 ng/ml).

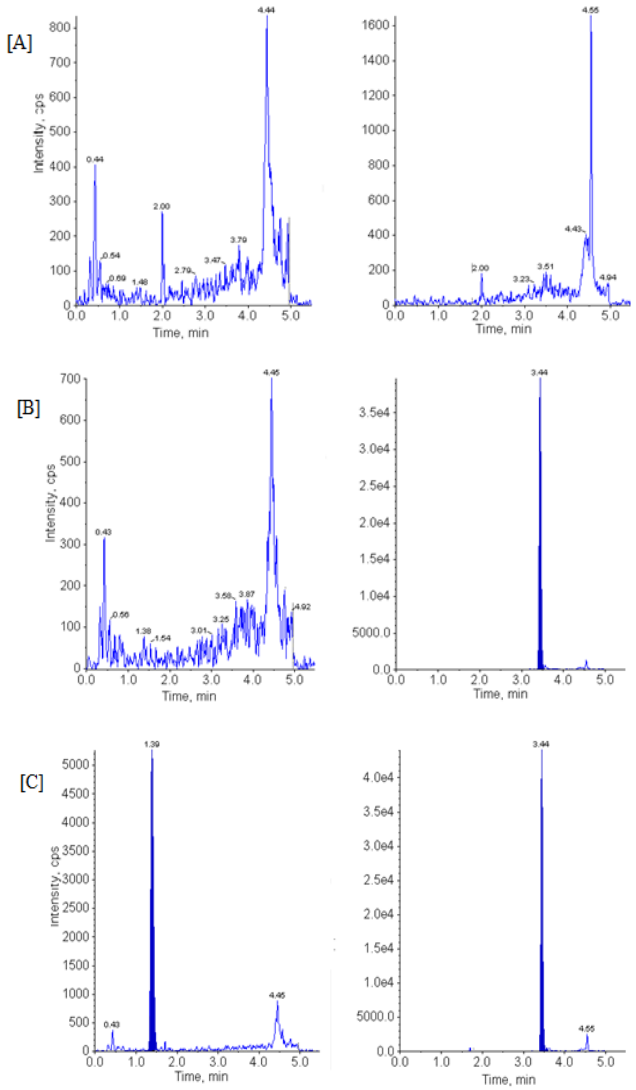

Fig. 2: Typical chromatogram of gemcitabine (left panel) and IS (right panel) in human blank plasma [A], plasma spiked with internal standard [B] and lower limit of quantification sample along with IS [C]

Recovery

Acetonitrile: water (90:10v/v) mixture as an extraction solvent proved to be rugged and provided the cleanest samples. The % recovery was more than 83.5% and the CV (%) was<4.0%.

Matrix effect and sensitivity

There was no significant matrix effect observed in all six lots of human blood for the analyte at low and high QC level concentrations. The precision and accuracy for gemcitabine at LQC concentration were found to be 12.01% and 99.07%, and at HQC level they were 11.1% and 93.67%, respectively, the %CV of the IS-normalized matrix factor was less than 12.0%. Results revealed that no significant matrix effect was observed in all the six batches of human blood.

The lowest limit of reliable quantification for the analytes was set at the concentration of the LLOQ. The precision and accuracy of analyte at 5.0 ng/ml concentration was found to be 2.66% and 99.8%, respectively.

Linearity, Precision and accuracy

The ten-point calibration curve was found to be linear over the concentration range of 5-5000 ng/ml for gemcitabine. After comparing the weighting factor models at none, 1/X and $1/X^2$,the linear regression equation with weighting factor $1/X^2$ of the analyte to the internal standard concentration found to produce the best fit for the concentration–detector response relationship. The mean correlation coefficient of the weighted calibration curves generated during the validation was ≥ 0.99.

The results for intra–day and inter–day precision and accuracy are summarized in table 1. The intra–day and inter day precision deviation values were all within 15% of the relative standard deviation (RSD) at low, middle 1, middle 2 and high quality control levels, whereas within 20% at LLOQ QCs level. The intra–day and inter–day accuracy deviation values were all within 100±15% of the actual values at low, middle 1, middle 2 and high quality control levels, whereas within 100±20% at LLOQ QCs level. The results revealed good precision and accuracy.

Dilution integrity

Dilution integrity was accessed at a concentration approximately 1.6 times of ULOQ. 50 µl of whole blood containing gemcitabine at a concentration of 8000 ng/ml was spotted on each circle of FTA DMPK-A cards. The samples were dried and extracted as previously described. The extracts were diluted 2x and 4x using blank DBS spots containing internal standard similarly extracted in the same batch.

Table 1: Intra and inter day precession and accuracy for the determination of gemcitabine in spiked human blood

Sample	Conc Added (ng/ml)	Intra-day Precision and Accuracy (n=12; 6 from each batch)			Inter-day Precision and Accuracy (n=30; 6 from each batch)		
		Conc. found mean±SD (ng/ml)	Precision (%CV)	Accuracy (%)	Conc. Found mean±SD (ng/ml)	Precision (%CV)	Accuracy (%)
LLOQ	5.2	5.230±0.216	4.14	100.5	5.256±0.245	4.67	101.0
LQC	14.5	16.178±0.750	4.64	111.5	16.0453±0.695	4.34	110.6
MQC1	245.1	233.429±10.717	4.59	96.6	233.635±7.730	3.31	96.7
MQC2	1050.0	1200.878±45.885	3.82	114.3	1144.982±65.884	5.75	109.0
HQC	4200.0	4101.566±179.257	4.37	97.6	4019.897±199.846	4.97	95.7

.Stability studies

Table 2 shows the Autosampler stability of the sample extracts stored at 4 °C for 72 hours. Post preparative stability for 62 h. Gemcitabine remained stable in DBS when stored for 3 months at room temperature.

Effect of blood volume spotted on the DBS card

Gemcitabine concentration from spot volumes of 20, and 30 µl was evaluated for demonstrating whether an exact spotting volume is required for accurate results. Table 3 shows both the spot volumes were comparable to those 50 µl with %CV of ≤11.8 and within 100.2 to 107% of nominal values.

Effect of hematocrit

Hematocrit values differ in cancer patients so attempts were made to establish the effect of hematocrit on DBS analysis. Table 4 shows that results with different hematocrit values ranging from 0.25% and 0.62% are within the acceptable limit.

Table 2: It shows stability data for gemcitabine in human blood

Stability test	QC spiked concentration (ng/ml)	Mean ± SD (ng/ml)	Precision (%CV)	Accuracy/ Stability (%)
Auto Sampler[a]	14.5	0.132±0.001	1.23	98.0
	4200	7.092±0.038	0.54	91.8
Re injection[b]	14.5	13.700±0.876	6.40	102.5
	4200	3875.433±202.401	5.22	101.4
Long term[c]	14.5	15.969±0.933	5.84	110.1
	4200	4060.011±247.316	6.09	96.6

[a]after 72h at 2–8 °C; [b] after 62 h at 2–8 °C; [c] after 90days at room temperature

Table 3: It shows effect of spotting volume on accuracy and precision of gemcitabine from extracted human blood

Sample	Spotting volume (µl)	QC Spiked concentration (ng/ml)	Mean ± SD (ng/ml)	Precision %CV	Accuracy %
1	20	14.5	15.466±1.817	11.75	106.6
		4200.0	4278.583±191.414	4.47	101.8
2	30	14.5	14.525±1.231	8.48	100.2
		4200.0	4414.013±201.008	4.55	105.1

Table 4: it shows the effect of hematocrit on accuracy and precision of gemcitabine from extracted human blood

Sample	Hematocrit	QC Spiked concentration (ng/ml)	Mean ± SD (ng/ml)	Precision %CV	Accuracy
1	0.25	14.5	13.485±0.874	6.49	93.0
		4200.0	4080.393±194.956	4.78	97.1
2	0.62	14.5	14.959±0.847	5.67	103.1
		4200.0	4008.649±183.023	4.57	95.4

Stability of gemcitabine in whole blood in presence and absence of THU: Comparision to DBS

Degradation of gemcitabine was observed in whole blood samples in an absence of THU. More than 22% of gemcitabine was deaminated in 3hr time. Fig. 3a shows presence of THU is needed for the exact quantitation of gemcitabine in plasma.

Interestingly, on the other hand the samples prepared on DBS cards with or without THU didn't make any effect in quantifying gemcitabine. Fig. 3b shows that gemcitabine was stable on DBS cards for nearly 90days.

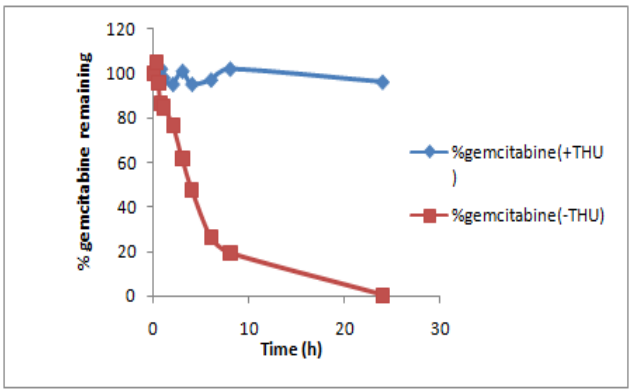

Fig. 3a: It shows Whole Blood Stability of gemcitabine in presence and absence of THU

DISCUSSION

In summary, we have developed a simple protein precipitation extraction in conjunction with a five minute LC methods which provided the efficiency required for a high throughput assay.

Compared with the proposed methods (Free man,KB *et al.* [11] 18 min, Keith,B *et al.* [13] 17 min, Yilmaz,B *et al.* [14] 20 min, Tashinga E. Bapiro *et al.* [21], 15 min) this method has the shortest run time. Lin,NM *et al.* [15] have used a higher volume of plasma(1 ml), R Honey well *et al.* [18] followed a liquid-liquid extraction technique with two columns which are connected serially to separate dFdC and dfdu.

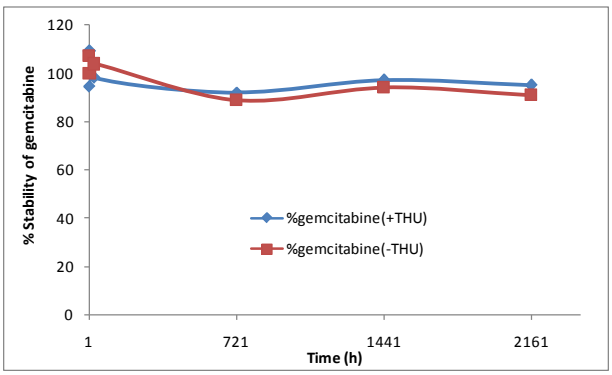

Fig. 3b: It shows Whole Blood Stability of gemcitabine in presence and absence of THU on DBS

Both these methods were able to quantify dfdu with a limit of quantification of 50 ng/ml. There is only a single method proposed with a shorter run time of 1.5 min proposed by Chester Bowen *et al.* [23]. But the limitation of this method was a tedious derivatization process with densyl chloride followed by a liquid liquid extraction of densyl derivatives from plasma. In the developed method we have used a very small volume of blood on DBS cards, followed by a simple protein precipitation extraction to get a sensitivity of 5 ng/ml, the lowest concentration reported compared to the published literature, except for the method proposed by Yan xu *et al.* [20], where they used a solid phase extraction technique to achieve a sensitivity of 5 ng/ml with 15 min run time. Tetrahydrouridine a cytidine deaminase inhibitor is not a stable compound and need to be added into sampling device at day of sampling, making it more difficult for the clinical staff to prepare it fresh each time and

pipetting out into sampling tubes. Experiments shown that the need of THF as stabiliser to inhibit the cytidine deaminase activity in blood or plasma. A degradation of parent compound to 22% was observed in negative control samples (without THF in blood samples) in 2hr. The method optimised by D'Arienzo CJ *et al.* [34] for stabilisation of prodrugs on DBS cards without addition of esterase inhibitors was an important reference for the method development in this study. The long term stability data for gemcitabine on DBS cards without THU proves that this novel method can be applied for sample collection and storage in analyzing the clinic samples.

CONCLUSION

The proposed method has several advantages like avoiding THU in blood tubes potentially minimises errors in the clinic during sample collection, reduces biohazard risks and experiments proved that samples are stable at room temperature for longer times can circumvent the need of freezers compared to other blood based samples. Despite that the method is simple and rapid with a short run time of 5 min compared to the published methods. This method can be applied in clinic for therapeutic drug monitoring and also in bioequivalence studies.

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CONFLICT OF INTERESTS

There are no conflicts of interest

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