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Original Article

DEVELOPMENT AND VALIDATION OF A RAPID AND SIMPLE REVERSED-PHASE HPLC METHOD FOR THE DETERMINATION OF GEMCITABINE IN HUMAN PLASMA

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ABSTRACT

Objective: In order to investigate the human plasma pharmacokinetics of dFdC, the objective of this work was to optimize and validate a rapid reversed-phase (RP) high-performance liquid chromatography (HPLC) method according to the guidelines of the international regulatory institutions: European Medicines Agency (EMA), Food and Drug Administration (FDA) and International Conference on Harmonization (ICH).

Methods: Chromatographic runs were performed on a RP-ACE-C18 column. Mobile phase was constituted of sodium acetate buffer (pH 5) and acetonitrile, in gradient mode, at a flow rate of 1 mL/min. Gemcitabine and cytarabine (internal standard) were detected at 290 nm.

Results: The method was shown to be selective, linear in the range of 0.25–10 mg/L (R^2=0.9998), accurate and precise within-run and between-run as reflected by the coefficient of variation values (<15%) and the relative errors values (<15%), stable and robust to changes in the column temperature and detection UV wavelength. Detection limit and lower limit of quantification were 0.22 and 0.25 mg/L respectively. **Conclusion:** The developed method is useful to measuring gemcitabine plasmatic concentrations in pharmacokinetics studies and in therapeutic drug monitoring.

Keywords: Gemcitabine, Cytarabine, Reversed-phase HPLC, Plasma.

INTRODUCTION

Gemcitabine (dFdC, 2',2'–difluorodeoxycytidine) is a deoxycytidine nucleoside analogue of deoxycytidine (pyrimidine antimetabolite), with a wide spectrum of antitumor activities. It is broadly used for standard treatment in pancreatic cancer, tumors of the lung, breast, and bladder, renal cell carcinoma, and cancer of the biliary tract either as a single drug or in combination with other cytotoxic agents. It is one of the reference drugs used in combination chemotherapy of NSCLC[1-7]. Comparing with deoxycytidine, two fluorine atoms have been inserted into the deoxyribose ring (Figure 1) and like other nucleoside analogues, gemcitabine is a pro-drug: it is inactive in its original form, depending on the intracellular machinery to gain pharmacological activity [5,7,8].

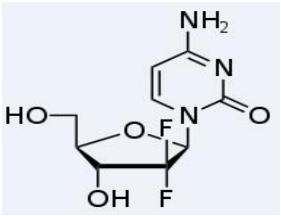

Fig. 1: Chemical structure of gemcitabine (2',2'-difluorodeoxycytidine)

Gemcitabine cellular influx occurs via de nucleoside transport system and undergoes intracellular phosphorylation by deoxycytidine kinase to form 2',2'-difluoro-2-deoxycytidine 5-monophosphate (dFdCMP), 2',2'-difluoro-2-deoxycytidine 5-diphosphate (dFdCDP), and 2',2'-difluoro-2-deoxycytidine 5-triphosphate (dFdCTP). dFdCDP and dFdCTP are responsible for the cytotoxic effects of gemcitabine. Gemcitabine is deaminated to the inactive metabolite 2',2'-difluorodeoxyuridine (dFdU) by cytidine deaminase [3,5,8-13].

Gemcitabine is usually administered at a dose of 1000 mg/m^2, by intravenous infusion, at a fixed dose rate of 10 mg/m^2/min, for 30 min, on days 1 and 8 of a 21 days treatment cycle. Previous studies suggested that the dFdCTP accumulation rate is saturated at a gemcitabine plasma concentration of 15–20 μM [4,11,12] and alternative doses and treatment schemes have been evaluated in order to optimize therapeutic efficacy of gemcitabine [9,11,12]. However, these schemes, basedon low infusion rates over prolonged time periods or ona low dose application of the drug, led to low plasmaconcentrations of gemcitabine and dFdU [11].

A recent systematic review and meta-analysis of all randomized controlled trials that compared the efficacy of doublet versus single third-generation cytotoxic agent as first-line treatment for elderly patients with advanced non-small-cell lung cancer (NSCLC) revealed that the use of gemcitabine in combination with other cytotoxic agents (doublet therapy) was superior to a single third-generation cytotoxic agent [14]. Nevertheless, drug dosage and treatment schedulesare still not optimized. Moreover, and although gemcitabine hydrochloride is considered as a relatively safe cytotoxic agent, caution is required due to the risk of induced lung injury and death[15]. These facts support the need of therapeutic drug monitoring for dosage adjustment or treatment discontinuation, as needed. The development and implementation of a simple and rapid assay to quantify gemcitabine plasmatic concentrationsin clinical practice will contribute to the individualization of gemcitabine treatments.

The quantification of gemcitabine plasmatic concentrationsusing an HPLC-UV method was previously described by others authors [3,9-13,16-18]. Nevertheless, we verified in our laboratory the existence of complex steps to the sample preparation, resulting in high costs and unnecessary time consumption. Further more the application of those protocols under our laboratory conditions resulted in non-satisfactory chromatograms, in terms of selectivity, accuracy and precision.

Therefore, the aim of the present work was to develop, optimize and validate sensitive, specific, accurate, precise, reproducible, robust and rapid HPLC-UV method to quantify dFdC, in human plasma samples, according to the guidelines of the international regulatory institutions European Medicines Agency (EMA), US Department of

Health and Human Services – Food and Drug Administration (FDA) and International Conference on Harmonization (ICH). This method will then be applied to the quantification of gemcitabine plasmatic concentrations to apply in therapeutic drug monitoring in patients with advanced NSCLC undergoing combination therapy of gemcitabine and carboplatin.

MATERIALS AND METHODS

Materials and reagents

Gemcitabine hydrochloride (2 -Deoxy-2′,2′-difluorocytidine; $C_9H_{11}F_2N_3O_4$·HCl) purity of ≥ 98% (HPLC) and Cytarabine crystalline (4-amino-1-β-D-arabinofuranosyl-2(1H)-pyrimidinone; ($C_9H_{13}N_3O_5$) (internal standard, IS) were purchased from Sigma Aldrich (Sigma-Aldrich Quimica, S. A., Sintra, Portugal). HPLC grade acetonitrile and methanol were purchased from Panreac Quimica Lda. (Cascais, Portugal) and triethylaminehydrochloride(TEA) ≥99% was acquired from Sigma Aldrich (Sigma-Aldrich Quimica, S. A., Sintra, Portugal). HPLC grade water was prepared in the laboratory using Milli-Q Ultrapure Water Purification System (Industrial Laborum Ibérica, SA, Braga, Portugal). All other reagents and solvents were of analytic or HPLC grade. Prior to use, mobile phase solvents were filtered using 0.45 µm filters and degassed in an ultrasonic bath for 15 min. Drug-free human plasma was obtained from healthy volunteers of the Porto Blood Regional Centre (Portuguese Blood Institute)and was used as a blank matrix.

Equipment

All HPLC runs were carried out using a LaChromUltra™ SystemAgilent (VWR International Lda, Carnaxide, Portugal), equipped with an organiser unit, two pumps L-2160U, a L-2200U auto-sampler, a L-2300 column oven, a L-2400 UV Detector and vacuum degasser. Results were acquired and processed with the EZ Chrom Elite™ Software (VWR International Lda, Carnaxide, Portugal). HPLC analysis was conducted with an ACE reversed-phase C18 column (ACE, Spain), with 5µm particle size, 4.6 mm internal diameters and 250 mm length.

Chromatographic conditions

Chromatographic analysis was performed in a gradient mode using two solvents (A and B). Solvent Aand solvent B consisted of sodium acetate bufferat pH 5 and acetonitrile, respectively. Initially, over 2 minutes, the run were performed by an isocratic mode of 98.5:1.5 (%A: %B) and then a linear gradient was ramped up from 1,5% to 7% of solvent B in 3 min,and was maintained for 4 minutes. To restore working conditions, a linear gradient was applied at the initial solvents proportion in 2 minutes and maintained for 2 minutes to ensure equilibrated analysis conditions in the next run. Total run time was 13 min and the mobile phase was pumped at a flow rate of 1 mL/min. Sample injection volume was 20 µl and the detection wavelength was 290 nm. All chromatographic runs were carried out in acolumn at 40ºC and samples placed in the auto-sampler at 4ºC and protected from light. Ratios of total gemcitabine peak area/total cytarabine (IS) peak area was used to quantify gemcitabine concentrations.

Preparation of stock and working solutions

A stock solution of 100 mg/L of gemcitabine hydrochloride was prepared by weighing 5 mg of gemcitabine into a 50 mL volumetric flask and making up to volume with methanol. Seven working solutions (2.5, 5, 10, 25, 50, 75 and 100 mg/L were obtained by measuring the required amount of the 100 mg/L stock solution a completing with a sufficient amount of methanol up to 1 mL A stock solution of 100 mg/L of cytarabine (IS) was prepared using 5 mg of cytarabine crystalline and methanol, making a 50 mL solution. All stock and working solutions were stored at 4ºC until use.

Preparation of standard solutions and quality controls

Standard solutions (calibrators) comprising 0.25, 0.5, 1, 2.5, 5, 7.5 and 10 mg/L were prepared in 1.5 mL polypropylene microtubes, by spiking 200 µL of blank human plasma with 100 µL of working solutions and 10 µL of IS stock solution.

Then 650 µL of methanol were added to each microtube. Samples were then vortexed for 1 minute and centrifuged during 15 min at 10500 rpm, at 4ºC. This step is taken to induce protein precipitation with the objective of eliminating potential interferences in the final chromatogram. After centrifugation, the supernatant (organic phase) is transferred to HPLC microvials for HPLC runs.

Quality controls (QC) were prepared similarly, with the respective working solutions (2.5, 7.5, 45 and 80 mg/L). Zero samples were prepared by adding 50 µL of IS(100 mg/L) and 750 µL of Methanol to 200 µL of drug-free plasma and blank samplesby adding 800 µL of methanol to 200 µL of drug-free plasma and the protein precipitation carried out as explained above. All standard solutions and quality controls were prepared in ice and protected from light. Some aliquots were stored at −70ºC for stability tests.

Validation methodology

Bioanalytical method validation is essential in drug analysis because this process ensures that the analytical procedure employed for the analysis is suitable for its intended use and show reliability of the results produced [19-21].

The current HPLC method was validated according to the EMA[21], FDA and to the ICH[20]guidelines.

Accordingly to these guidelines, to ensure the acceptability of the performance and reliability of the analytical results, the following characteristics were considered: selectivity, carry-over, lower limit of quantification (LLOQ), detection limit (DL), calibration range, accuracy, precision, stability of the analyte in the biological matrix and stability of the analyte and of the internal standard in the stock and working solutions under the entire period of storage and processing conditions.

A seven-point calibration curve was constructed by plotting the ratio of gemcitabine/IS peak areas (y) *versus* gemcitabine concentration (x) (carried out in triplicate) The concentration range for the calibration curve (0.25 to 10 mg/L) was defined according to the expected gemcitabine plasma concentrations [16,17]. Results for blank samples were not used as part of the calibration curve [21]. Gemcitabine standard solutions concentrations were then back calculated,using the calibration curve, and mean accuracy values were determined.

Slope, intercept and linearity were determined by calculating the regression equation from the plot of peak area ratio *vs* concentration, for seven standard solutions (0.25, 0.5, 1, 2.5, 5, 7.5 and 10 mg/l) using the linear least squares method [20], and by analysis of the respective response factors (*i. e.* peak area ratio divided by the concentration of each standard solution) [22,23].

The **selectivity** was demonstrated by the analysis of six blank plasma samples [21].

The **carry-over** was assessed by injecting of six blank plasma samples. After the injection of a high concentration standard solution (upper limit of quantification; ULOQ) [21].

The **lower limit of quantification (LLOQ)** is the lowest concentration of analyte in a sample, which can be reliably quantified, with acceptable accuracy and precision. The LLOQ signal should be at least 5 times the signal of a blank sample [21].

The **detection limit (DL)** is determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected [20]. The DL of our method was calculated using the following equation: **DL = (3.3 σ)/ S**, where σ is the standard deviation of the mean value resulting from analysis of an appropriate number (N=20) of blank samples and S is the slope of the calibration curve [20].

The **accuracy** was tested by calculating the percentage of the nominal value (relative error %) of four different gemcitabine concentrations (QC). The four QC used were (0.25, 0.75, 4.5 and 8.0 mg/L). Accuracy was evaluated within a single run (within-run accuracy) and in different runs (between-run accuracy). Within-run accuracy was determined by analyzing, in a single run, a minimum of

5 samples per QC level. For the validation of the between-run accuracy, QC samples, from at least three runs, were analyzed on three different days [21,24].

The **precision** of the analytical method describes the closeness of repeated individual measures of analyte expressed as the coefficient of variation (CV) [24]. For precision determination, the QC samples were analyzed within a single run and in different runs, using the same runs and data as for the demonstration of accuracy [21].

Robustness was evaluated by deliberately varying the column temperature (± 5°C) and the detector wavelength (± 3 nm) [20,24,25].

Method applicability

An analytical run consists of the blank sample (processed matrix sample without analyte and without IS) and a zero sample (processed matrix with IS), 6 calibration standards, 4 levels of QC samples (in duplicate), and study samples to be analyzed. As indicated before the calibration standards and QC samples should have been spiked independently using separately prepared stock solutions [21]. All samples were analyzed in triplicate, except when indicated otherwise. The optimized and validated method was used to quantify gemcitabine plasmatic concentrations in our study population to perform a pharmacokinetic study and to develop a pharmacokinetic/pharmacodynamic model to dose individualization in treatment scheme carboplatin/gemcitabine of NSCLC patients.

RESULTS AND DISCUSSION

Method development and optimization

Prior to the validation step, the hereby-proposed method was developed to provide a simple and optimized procedure, with reduced time and analysis costs. For instance, we simplified some aspects related to sample preparation, proceeding to a one-step only protein precipitation. Moreover, several chromatographic performance parameters were considered, specifically, peak resolution (*R*), peak symmetry (described by the tailing factor, *T*) and the number of theoretical plates (*N*)[19,22,24].

Initial runs were performed using similar mobile phases and internal standard as those used by other authors [3,9-13,16-18]. With the initial use of a mobile phase (MP) made up with phosphate buffer, pH 3/acetonitrile at a 98:2 ratio, phosphate crystals precipitation was observed. Therefore, to prevent collapse and damage of the column we substituted phosphate buffer pH 3 by sodium acetate buffer pH5, in the MP. This new MP was tested at various proportions, ranging from 90:10 to 99.5:0.5, using various flows and gradients.

The gradient varying between 98.5:1.5 and 93:7 (Section 2.3 Chromatographic conditions) was the most acceptable combination for the MP, allowing best peak resolution (>2) from the matrix compounds and good peak symmetry, as indicated by the obtained tailing factor values (0.8–1.2). These values are internationally accepted and indicate good method performance [19,24]

In columns packaged with high silanol activity silica, the use of triethylamine (TEA) in MP remains a crucial step to improve peak symmetry [22,24,26]. Theoretically this addition can inhibit, or at least reduce, gemcitabine and cytarabine interaction with available acidic silanols, once these molecules contain amine groups in their chemical structure [22,24]. However, the column we used (ACE C18) has few acidic silanols available to cause peak tailing. These stationary phases are typically made with low activity silica (high purity "type B" silica) and have their surface highly covered by the bonded phase [26]. As such, in our working conditions, TEA was not added because this would probably provoke a new interfering peak in the chromatogram.

Initially adenine was tested as the internal standard (IS). However, the poor chromatographic retention on reversed phase columns revealed to be a critical problem. Furthermore, we couldn't separate adenine, with adequate resolution, from the solvent and from the gemcitabine peak. Therefore, we decided to substitute cytarabine by adenine and good resolutions (>2)were then obtained both for gemcitabine and solvent. The resulting chromatographic performance parameters of the chosen setup for validation are presented in Table 1.

Table 1: Chromatographic performance parameters of the chosen setup

Chromatographic parameters	Result[a]	Acceptance criteria
Gemcitabine retention time (min)	8.33±0.05	-
Cytarabine retention time (min)	5.27±0.05	-
Gemcitabine resolution, *R* G	8.73±0.50	> 2
Cytarabine resolution, *R* IS	2.48±0.08	> 2
Gencitabine Tailing factor, *T* G	1.03±0.10	0.8 - 1.2
Cytarabine Tailing factor, *T* IS	1.01±0.04	0.8 - 1.2
Gemcitabine Number of theorical plates, *N* G	18522.57±4770.89	N > 2000
Cytarabine Number of theorical plates, *N* IS	2273.75±240.65	N > 2000

[a] Presented as mean value ± standard deviation (SD).

Although the maximum absorption of gemcitabine has been reported to occur at 272 nm[27], as confirmed by others authors [10,11,17,18], we tested a UV wavelength range between 250 and 300 nm. The best detection was verified at a wavelength of 290 nm, where the noise signal was in fact small. The highly polar nature of the analytes causes a rapid elution from reversed-phase columns [24] even with a very low (1%) organic content in the MP.

Therefore, several columns were tested (ACE C18 (250x4.6 mm, 5 µm particle size), Symmetry C8 (250x4.6, 5 µm particle size) and Waters Spherisorb® C18 ODS2 (150x4.6, 5 µm particle size)) and theACE C-18 column appeared to be the most suitable for use in this method.

Method validation

Selectivity

The method selectivity was evaluated by comparing the chromatograms of six blank plasmas to the chromatogram of the calibrator used to determine the LLOQ (0.25 mg/L) and to the chromatogram of the stock solution of cytarabine (50 mg/L). No peaks at the retention time of gemcitabine (8.33 min) and cytarabine (5.27 min) were observed (fig. 2) and a representative chromatogram showing the separation of the analytes from the matrix is shown in fig. 3.

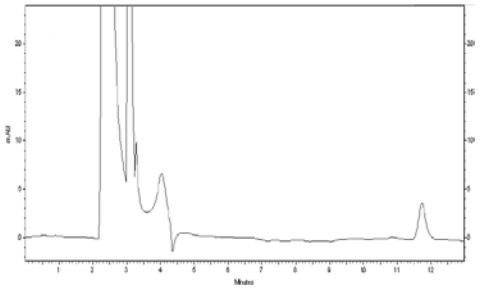

Fig. 2: Representative chromatograms of human blank plasma.

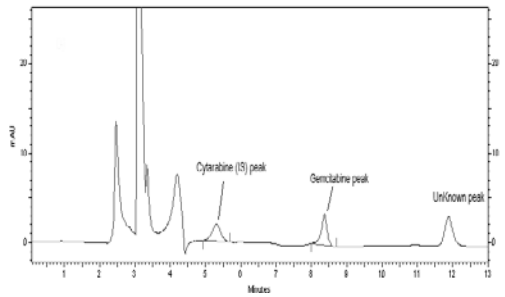

Fig. 3: Representative chromatograms of human plasma with internal standard (IS) and gemcitabine standard solution 5mg/L.

The peak observed at 12 minutes is resultant of the matrix. Most probably being plasmatic proteins since that peak was also present in the blank plasma samples. As acceptance criteria, the absence of interfering components was validated when the signal is lower than 20% of the LLOQ for the analyte and 5% for the IS. The results obtained are presented in table 2. Overall, obtained data provides evidence that the method can be regarded as selective since no potential interfering peaks were observed.

Carry-over

Carry over was not detectable in six blank samples, analyzed after the higher concentration standard solution. Carry over absence criterion: noise signal of the blank plasma should be lower than 20% of the LLOQ and lower than 5% of the IS. Obtained results are presented in table 3.

Table 2: Gemcitabine and cytarabine selectivity data

Gemcitabine Selectivity				
	LLOQ (0,25mg/L) area	20% of LLOQ area	Blank area	Result
Mean Value (N=6)	5167.50	1033.50	657	< 20%
Cytarabine (Internal Standard) Selectivity				
	IS area	5% of IS area	Blank area	Result
Mean Value (N=6)	129196.33	6459.82	16	< 5%

LLOQ: Lower Limit of Quantification

Table 3: Gemcitabine and Cytarabine carry-over data

Gemcitabine Carry-over				
	LLOQ (0,25mg/L) Area	20% of LLOQ Area	Blank Area	Result
Mean Value (N=6)	5167.50	1033.50	627.17	< 20%
Cytarabine (Internal Standard) Carry-over				
	IS Area	5% of IS Area	Blank Area	Result
Mean Value (N=6)	129196.33	6459.82	25.17	< 5%

LLOQ: Lower Limit of Quantification

Lower Limit of Quantification (LLOQ) and Detection Limit (DL)

The lowest concentration at which an analyte can be detected (DL) or quantified with adequate accuracy and precision (LLOQ) can be determined by different approaches [20]. In the present study, the LLOQ was firstly defined according to the expected plasmatic concentrations. As the acceptance criterion for LLOQ gemcitabine signal, that should be at least 5 times the signal of a blank sample[21]. The DL was calculated from the standard deviation (SD) of the mean value obtained from the analysis of 20 blank samples and the slope of the calibration curve, as previously described (2.7. Validation Methodology). LLOQ and DL were defined as 0.25 and 0.22 mg/L, respectively.

Calibration range

In the construction of the calibration curve, seven standard solutions (0.25, 0.5, 1, 2.5, 5, 7.5 and 10 mg/L) were analyzed: four different runs carried out in triplicate, in four consecutive days. The linear regression of the ratio of the analyte area to that of IS, versus gemcitabine concentration was used. The mean equation (N=4) of the calibration curve obtained from media of those seven points was

1) $y = 0.1599(\pm 0.0023)X - 0.019\ (\pm 0.0117).\ R^2 = 0.9998$

Where y is the *ratio* gemcitabine peak area /IS peak area and X is the gemcitabine concentration in mg/L. Mean and SD values for *y* and *X* are indicated inside brackets.

Linearity

Firstly, linearity was studied in the concentration range of 0.25 – 10 mg/L by visual inspection of the calibration curve plotting (fig. 4) and then by calculating the coefficient of determination (squared correlation coefficient: R^2)by the least squares method[23]. Good linearity was considered whenR^2 higher than 0.999 were obtained, accordingly to what is internationally accepted[19,24]. In addition, linearity of the regression line was evaluated by a procedure based on the residual sum of squares: taking the regression line as the mean, a RSD was calculated for all data points, revealing values <2.0%, respectively a mean value of 0,90%, as internationally accepted [19,25].

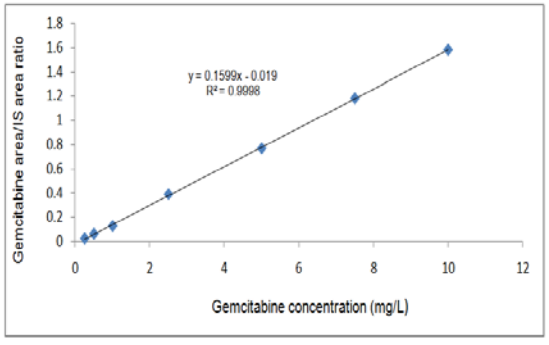

Fig. 4: Linearity studies for the proposed HPLC method: calibration curve obtained with gemcitabine standard solutions (N=7)

Although these are very practical ways of evaluating linearity data, they are not true measures of linearity. The coefficient of correlation can be subject of misinterpretation and may give a misrepresentation of linearity, since different datasets can yield

identical regression statistics and should be reinforced by complementary linearity evaluation methods [19,22,25].

As such, an analysis of response factors (*ratio* gemcitabine peak area /IS peak area divided by the concentration of each standard solution) for the proposed range was also carried out. The visual inspection and linear regression by the method of least squares of the plot of the response factors versus concentration of standard solutions (fig.5) revealed a near zero slope (0.0042), thus reinforcing the evaluation of the method as linear [22,25].

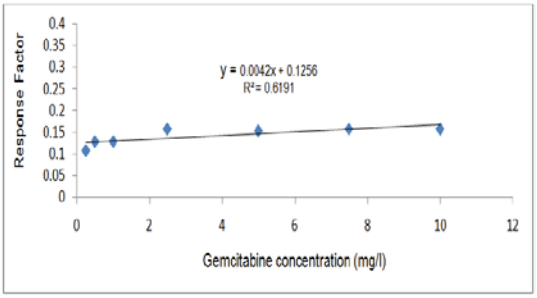

Fig. 5: Linearity studies for the proposed HPLC method: response factor versus gemcitabine standard solutions concentration (N=7).

Back Calculated Concentrations

The back calculated concentrations, represents the first point of control during the bioanalytical method validation. This procedure, involving the standard solutions is useful to validate or reject some points of the concentration range used. In the table 4 are indicated, in terms of accuracy the relative error(RE%) of the back-calculated concentrations of the standard solutions used in the calibration curve. All back calculated concentrations satisfied the acceptance criterion: ±15% of the nominal value, except for the LLOQ for which it should be within ±20% interval; at least 75% of the standard solutions must fulfill this criterion[21].

Table 4: Standard solutions back calculated concentrations accuracy data

Theoric concentration (mg/L)	Media (N=4)	RE (%)	Result
0.25	0.30	-18.12	<20%
0.5	0.52	-4.03	<15%
1	0.94	6.12	<15%
2.5	2.57	-2.76	<15%
5	4.82	3.69	<15%
7.5	7.41	1.25	<15%
10	10.03	-0.28	<15%

Accuracy

Accuracy is the closeness in agreement of the nominal true value to the determined value obtained by method developed, by calculating the percentage recoveries of the mean concentration of the analyte at four different concentrations. The four quality controls (QC) (0.25, 0.75, 5 and 7.5 mg/L) were carefully prepared in the same way standard solutions were.

In table 5results for the within run accuracy (N=5) and in table 6results for the between-run accuracy (N=3) shows close agreement between experimental and nominal values, fulfilling the internationally recognized acceptance criterion: the mean concentration should be within 15% of the nominal values for the QC samples, except for the LLOQ which should be within 20% of the nominal value [21].

Precision

Method precision is a measure of the ability of the method to generate reproducible results. Precision was validated for within-run and between-run, in terms of coefficient of variation (CV, %) as demonstrated in table 5 and 6, respectively. These results indicate that the developed and optimized HPLC method presents good precision, since it fulfills the internationally recognized acceptance criterion: CV values should not exceed 15% for the QC samples, except for the LLOQ which should not exceed 20%[21].

Table 5: Within-run Accuracy and within-run Precision data

QC (mg/L)	Media measured concentration (mg/L) (N=5)	RE(%)	Result	CV (%)	Result
0.25	0.28	-11.69	<20%	8.66	<20%
0.75	0.72	3.74	<15%	3.74	<15%
4.5	4.24	5.76	<15%	1.6	<15%
8	7.77	2.83	<15%	2.57	<15%

QC: Quality control; RE: Relative error; CV: Coefficient of variation

Table 6: Between-run accuracy and between-run precision data

QC (mg/L)	Media measured concentration (mg/L) (N=3)	RE(%)	Result	CV (%)	Result
0.25	0.29	-16.51	<20%	6.52	<20%
0.75	0.72	3.91	<15%	3.52	<15%
4.5	4.19	6.95	<15%	0.78	<15%
8	7.87	1.59	<15%	3.41	<15%

QC: Quality control; RE: Relative error; CV: Coefficient of variation

Stability

The stability of gemcitabine and IS was tested under conditions similar to those that would be employed for the sample in a real time analysis.

Following the validation requirements for bioanalytical methods, we evaluated: the stability of stock solutions (gemcitabine and cytarabine) during 24h over analysis conditions (in auto-sampler at 4ºC and protected from light), and also was evaluated the mobile phase stability after 1 week at room temperature. Since, cytarabine cannot be stored at room temperature (storage temperature: 2-8ºC), the implementation of this test in our working conditions, have not applicability.

To validate the storage conditions of the real samples, was evaluated the stock solutions stability frozen at −70ºC over 4 weeks, and refrigerated at 4ºC over 4 weeks, by preparing working solutions from the stock solutions stored at those conditions. At the respective times, these tests were performed by preparing in triplicate the lower (0,25mg/l) and the higher (8 mg/l) QC. Accuracy results (**table 7**), indicated as RE(%),demonstrates that the HPLC method developed shows good stability and can be used to analyze fresh and frozen samples, once it fulfilled the international acceptance criteria (RE%<2%) [19]. Other authors have also demonstrated gemcitabine stability when stored at room temperature up to 6 days[3,9] and at −70ºC for up to 2 years [9].

Table 7: Stability results for gemcitabine and cytarabine stock solutions by preparing the lower and the higher quality controls.

Stock solution stability refrigerated at 4º over 1 month		
QC	Media (N=3)	RE %
0.25	0.25	1.73
8	8.05	-0.62
Stock solution stability frozen at -70ºC over 1 month		
QC	Media (N=3)	RE %
0.25	0.25	0.37
8	7.93	0.82
Sample stability at the auto-sampler working conditions at 4ºC over 24h		
QC	Media	RE %
0.25	0.25	-0.30
8	7.96	0.45
Mobile phase stability at room temperature over 1 week		
QC	Media	RE %
0.25	0.25	-1.58
8	7.98	0.31

Robustness

Robustness is a measure of the performance of a method to remain unaffected when small and deliberate changes are made. This characteristic provides an indication of the method reliability during normal usage. The intent of robustness validation is to identify critical parameters for the successful implementation of the method. Robustness was partially evaluated during method development, when important conditions (e. g., peak shape, sensitivity) was optimized to improve method performance[20,22,25]. In this study, we tested deliberated variations in column temperature (±5°C) and in detect or wavelength (±3 nm). These method variations did not reveal differences greater than ±15% of the nominal value for quality controls tested when method working conditions changes ± 5ºC in oven temperature and with detection at 287 nm (-3 nm) (table 8). Relatively to peak characteristic, namely resolution, tailing factor and the number of theoretical plates were similar to the initial and validated conditions, respectively 4.16, 1.03 and 2648 to cytarabine peak and 8.73, 1.05 and 21768 to gemcitabine peak. On the other hand, when varying the wavelength detection to 293, we obtained unacceptable relative errors (around 20%) as reflex of poor peak.

At this wavelength we verified a drastically variation on area ratios and we conclude that the absorption of gemcitabine decrease disproportionally to cytarabine. Furthermore, 290 nm is a great wavelength to eliminate interfering compounds but reduces significantly the method sensibility.

Table 8: Robustness tests data.

Robustness – Oven temperature at 35ºC and detection at 287 nm		
QC (mg/l)	Media (N=3)	RE %
0.25	0.28	-10.63
0.75	0.78	-4.28
4.5	4.58	-1.72
8	8.05	-0.73
Robustness – Oven temperature at 45ºC and detection at 287 nm		
QC (mg/l)	Media (N=3)	RE %
0.25	0.25	1.78
0.75	0.76	-1.39
4.5	4.55	-1.18
8	8.21	-2.62

CONCLUSION

The bioanalytical method described, based on arapid and simple sample preparation (protein precipitation) and HPLC-UV determination, allows the quantification of gemcitabine in human plasma. The optimized RP-HPLC method was fully validated according to the EME, FDA and ICH guidelines. It was demonstrated to be selective, linear, accurate, precise, stable and robust in the range of 0.25–10 mg/L. Also, the DL and the LLOQ were determined. The method represents a suitable and useful tool for the analysis of gemcitabine plasmatic concentrations in pharmacokinetic/ pharmacodymanic studies in NSCLC patients to perform dose individualization of the treatment schemes.

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CONFLICT OF INTEREST STATEMENT

The authors report no declarations of interest.

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