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Non-invasive quantification of 5 fluorouracil and gemcitabine in aqueous matrix by direct measurement through glass vials using near-infrared spectroscopy

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ABSTRACT

Fourier transform near infrared spectroscopy (NIRS) was used for quantitative analysis of two cytotoxic drugs used in pharmaceutical infusion, 5-fluorouracil (5FU) and gemcitabine (GEM), at therapeutic concentrations in aqueous matrix.

Spectra were collected from 4000 cm^{-1} to 13,000 cm^{-1} by direct measurement through standard glass vials and calibration models were developed for 5FU and GEM using partial least-squares regression. NIR determination coefficient (R^2) greater than 0.9992, root-mean-square-error of cross-validation (RMESCV) of 0.483 mg/ml for 5FU and 0.139 mg/ml for GEM and the root mean square error of prediction (RMSEP) of 0.519 for 5FU and 0.108 mg/ml for GEM show a good prediction ability of NIR spectroscopy to predict 5FU and GEM concentrations directly through a glass packaging. According to accuracy profile, the linearity was validated from 7 to 50 mg/ml and 2 to 40 mg/ml for 5-fluorouracil and gemcitabine respectively.

This new approach for cytotoxic drugs control at hospital has shown the feasibility of near infrared spectroscopy to quantify antineoplastic drugs in aqueous matrix by a direct measurement through glass vial in less than 1 min and by non-invasive measurement perfect to limit exposure of operator to cytotoxic drugs.

1. Introduction

According to World Health Organization, cancers represented the first cause of death with more than 12.7 million of peoples new cases worldwide diagnosed in 2008 [1]. Cytotoxic drugs represent the most often used in anticancer chemotherapy treatment.

The treatment is adapted for each patient and concentrated formulations have to be diluted by nurses or pharmacy technicians with chloride sodium 0.9% or glucose 5% to obtain individualized treatment in accordance to prescriptions. Even if final control of cytotoxic preparations is not required by pharmaceutical regulations, analytical control reduces medication errors and thus, consequences on patient health. By identification and quantification, analysis control can ensure correct molecule and concentration and contributes to improve the security of the antineoplastic drugs process at hospital. Numerous analytical methods such as HPLC/UV, LC/MS/MS, GC/MS have been developed to quantify cytotoxic drugs in pharmaceutical formulations [2].

At hospital, cytotoxic drugs are identified but also quantified using flow injection analysis coupled with a diode array detector (FIA-UV) using UV absorption properties [3,4]. Thus, at the end of the cytotoxic preparation process, a sample of each preparation was collected for analytical control.

However, exposure to antineoplastic drugs can cause short-term toxicity such as nausea, rush but also long term effects with fecundity troubles and organ toxicity because of potential genotoxic, carcinogenic, teratogenic properties. Despite guidelines for good handling of cytotoxic drugs [5], cytotoxic drugs have been identified in urine samples of health care workers [6–11]. Thus, handling those drugs presents a risk of occupational exposure for health care workers during preparation, administration but also control of those chemotherapy drugs.

In this context, non-invasive techniques have to be preferred to control antineoplastic preparations to minimize this occupational exposure. Due to its rapidity, non-invasive and non-destructive properties, near infrared spectroscopy (NIRS)

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represent an interesting method. This method does not require any sample preparation, and thanks to a short acquisition time, allows a high measurement throughput for a large number of molecules which can be quantified [12].

Numerous methods have been developed with NIRS to determine active content such as active drugs, excipients or moistures in various types of pharmaceutical formulations (*i.e.* powder, granulate, tablet, gel, lyophilized vials and liquid) [12–15].

Because of the possibility of rapid, non-destructive and non-invasive analysis, the use of NIRS has recently increased in industry and extended at hospital to control pediatric capsules [16]. NIRS is now currently used for process analytical technology (PAT) in accordance to Pharmaceutical Current Good Manufacturing Practices to control raw materials, intermediate but also final products [17].

However, due to the high absorption of water in the NIR region, the determination or the quantification of chemical molecule in aqueous environment seems to be very difficult. This explains the non-popularity of NIRS to quantify chemical component in aqueous liquid formulations. In fact, the NIR absorption is due to combination bands of the chemical component, the vials and the vector. Whereas specific wavelengths are proportional to the concentration of chemical components, NIR spectra are very complex and complicated to interpret [18]. Broad *et al.* have shown the possibility to quantify in a multi-component pharmaceutical oral liquid by direct measurement through amber plastic bottles using Fourier transform near-infrared spectroscopy (FT-NIRS) [19].

Thus, the aim of this pilot study was to assess the feasibility of near infrared spectroscopy as a non-invasive analytical method to quantify cytotoxic drugs at therapeutic concentrations in aqueous solution by direct measurement through glass vials.

2. Materials and methods

2.1. Reagents

Because 5-fluorouracil (5FU) and gemcitabine (GEM) (Fig. 1) are ones of the most often used cytotoxic drugs, those two molecules have been selected for this feasibility study. 50 mg/ml 5FU vials were obtained from Teva (La defense, France) and containing water with hydrochloric acid and sodium hydroxide. 40 mg/ml GEM vials with ethanol and water with hydrochloric acid and hydroxide sodium as excipients were obtained from Mylan (Saint Priest, France).

2.2. Experimental design

2.2.1. Calibration and validation sample sets

Solutions containing drug concentrations in the range from 1 to 40 mg/ml for GEM and 1 to 50 mg/ml for 5FU were independently produced by dilution of the respective commercialized solution into chloride sodium 0.9% (v/v) FreeFlex® (Fresenus Kabi, Louviers, France).

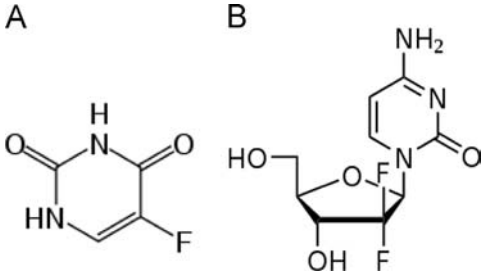

Fig. 1. The chemical structure of 5-fluorouracil (A) and gemcitabine (B).

To develop a robust calibration model, different sources of variability have been introduced into the models. For each drug, 5 series of solutions were prepared using 5 vials of cytotoxic drugs from the same batch by 5 operators. Each series included 10 and 11 levels of concentrations for GEM and 5FU respectively. Dilutions were produced using 5 batches of chloride sodium 0.9%. Due to direct measurements through the glass vial, the variability of the packaging had also been taking into account by introducing each solution into 3 different glass vials Interchim® of 2 ml (Montluçon, France). Thus, the sample set comprised 150 samples for GEM and 165 samples for 5FU.

All solutions were analyzed by FT-NIRS and the spectra were split into two groups: first, a calibration set including 3 series (90 samples for GEM and 99 samples for 5FU) to develop the prediction model and second, a validation set including 2 series (60 samples for GEM and 66 samples for 5FU) to evaluate the best prediction model in accordance to Guidelines on the use of NIRS [20].

2.2.2. Pharmaceutical preparation sample

In October 2012, a total of 58 pharmaceutical preparation samples from 2 to 7 mg/ml of 5FU and 40 samples from 2 to 6 mg/ml for GEM were collected from the production at the end of the cytotoxic preparation process. All samples were conditioned in glass vials Interchim®.

2.3. Instrumentation

2.3.1. NIR spectroscopy

NIR transmission spectra were analyzed using a Bruker Vector 33 SI001400 FT-NIR spectrophotometer (Bruker Optics®,Ettlingen, Germany) configured with a tungsten lamp source, a helium–neon 632.8 nm laser and a Ge diode detector. Spectral data were collected and analyzed using Opus software version 6.5 (Bruker Optics®, Ettlingen, Germany).

All spectra were collected by accumulation of 64 scans. Samples were scanned with a resolution of 8 cm^{-1} over the range from 4000 cm^{-1} to 13,000 cm^{-1}. An adaptation of the FT-NIR sample compartment has been done to align the vial and secure the position of the sample on the base plate. A glass vial Interchim® with 0.9% chloride sodium was used as a background reference.

2.3.2. Flow injection analysis with UV detector (FIA-UV)

FIA was performed using on Varian Pro Star HPLC system (Agilent technologies®, Les Ulis, France) equipped with automatic sample Prostar 410, a pump Prostar 230, a column valve module Prostar 500 and a diode array detector Prostar 330. All analysis were performed using Galaxie® software (Varian®, Les Ulis, France). 5 μl of 5-fluorouracil sample and 6 μl of gemcitabine sample were injected at room temperature across the chromatographic system without column in isocratic condition. The mobile phase was ultra-pure water from Milli-Q® integral water purification system (Millipore Guyancourt, France), with a flow rate of 1.5 ml/min. The DAD detector was used to monitor spectral data using a spectral range from 200 to 400 nm. The quantification was carried out at 269 nm and 268 nm for 5FU and GEM respectively. Each collected ultraviolet spectrum was compared with reference library for identification. Both for 5FU and GEM, the analytical method was validated from 1 to 10 mg/ml with a R^2 of 0.9981 and 0.9998 respectively.

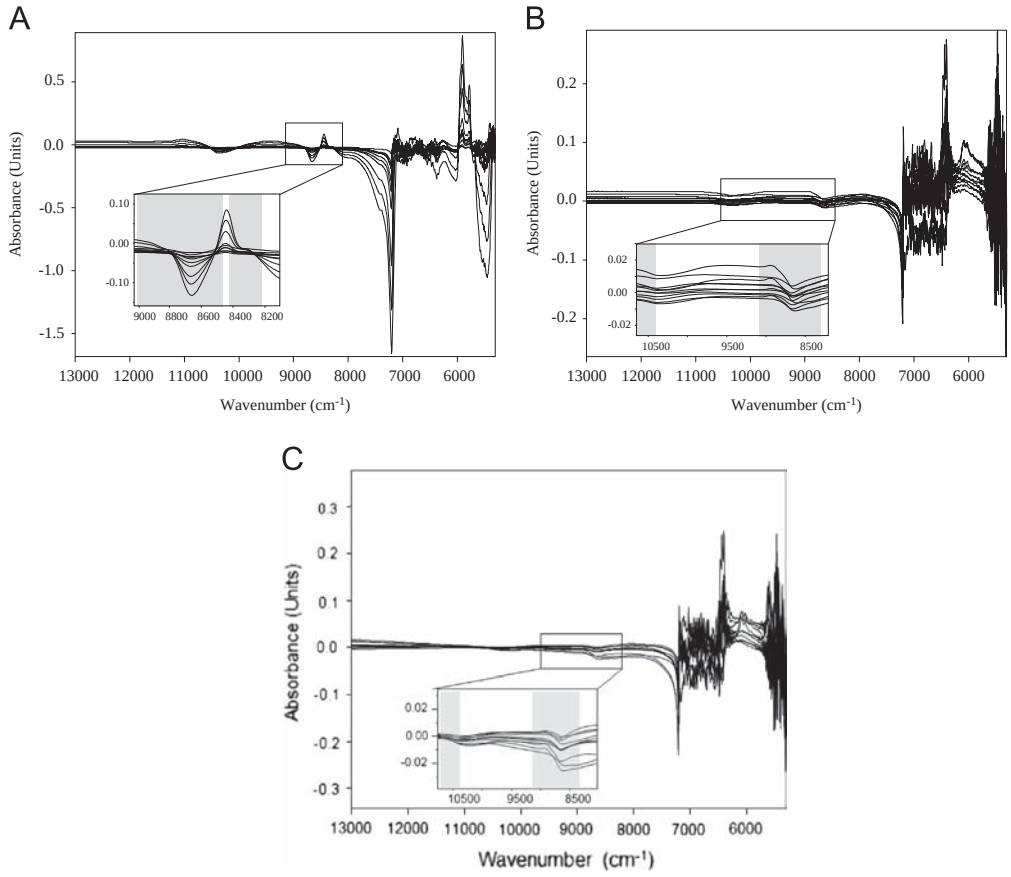

Fig. 2. NIR spectra of gemcitabine from 1 to 40 mg/ml without pretreatment (A) and 5-fluorouracil from 1 to 50 mg/ml without pretreatment (B) and after straight line subtraction (C).

2.4. Data analysis

2.4.1. Determination of calibration models

NIR signals content complex information and thus, required chemometric method to extract spectral relevant information and obtain quantitative information.

The calibration model was developed using leave-one-out cross validation (LOOCV) method coupled to partial least square (PLS) regression analysis. Different calibration models were investigated using first and second-order Savitsky-Golay derivatives, standard normal variate (SNV) or multivariate scatter correction (MSC), linear offset and straight-line subtraction.

To assess the error of prediction and validate the calibration model, validation set data were used as unknown samples and their concentration values predicted using the calibration model. The error of prediction was thus estimated across the root mean square error of cross validation (RMSECV), the root mean square error of calibration (RMSEC) and the root mean square error of prediction (RMSEP).

The optimal number of latent variables was determined for the lowest error of prediction in order to decrease the possibility of over fitting the model. Across all PLS models, the best calibration model was selected regarding RMSECV, RMSEP and correlation coefficient (R^2). All calculations were performed with Opus software version 6.5 (Bruker Optics®, Ettlingen, Germany).

2.4.2. Determination of calibration model performances

To complete this approach, the accuracy profile was established with NIR predicted data. Accuracy, precision, low limit of quantification (LLOQ), range of linearity, recovery and specificity were calculated to evaluate the performances of the calibration model.

2.4.3. Pharmaceutical preparation samples analysis

In accordance to NIRS guidelines [20], an external validation was conducted using samples from the production. Thus, pharmaceutical preparation samples of 5FU and GEM respectively were collected and both analyzed by NIRS and FIA UV methods. NIR 5FU and GEM predicted values were determined using calibration model previously developed and statistically compared to FIA UV predicted value by Bland Altman method conducted with XLSTAT® software.

3. Results and discussion

NIR analysis was performed through the container. Thus, NIR spectra are complex spectra resulting on the contribution of glass vial signal, pharmaceutical ingredient signal but also excipients.

To develop a predictive model, commercialized solutions were used for calibration and validation set samples. Thus, spectral data resulted of glass vial, excipients and active ingredient contributions. Although excipients may contribute to modify the environment of cytotoxic drugs and O-H vibrational band, excipients of 5FU, water with hydrochloric acid and sodium hydroxide, no contribute to spectral modification. In addition to water with hydrochloric acid and sodium hydroxide, the gemcitabine formulation included ethanol. A large contribution of ethanol to NIR spectrum of gemcitabine was observed.

To limit the contribution of glass of the vials and sodium chloride on spectral data, all analysis were performed using a

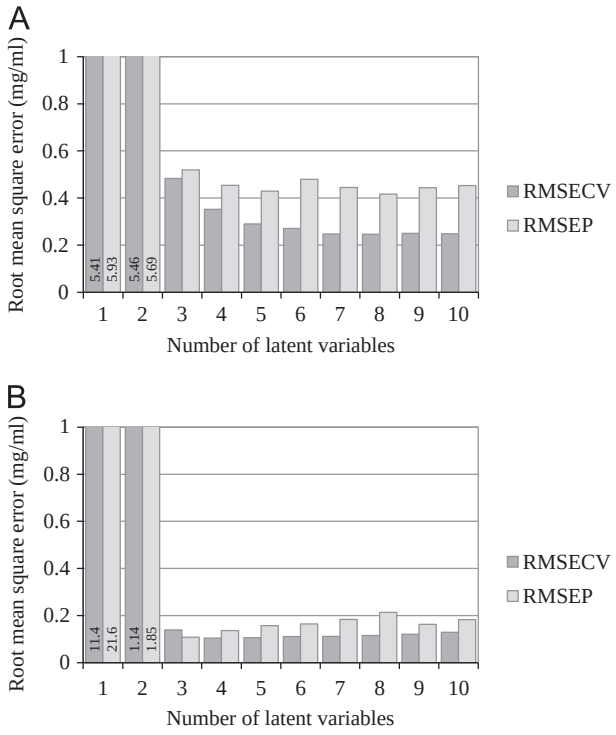

Fig. 3. Root mean square error of prediction (RMSEP) and root mean square error of cross-validation (RMSECV) according to the number of PLS latent variables for 5FU prediction model (A) and GEM prediction model (B).

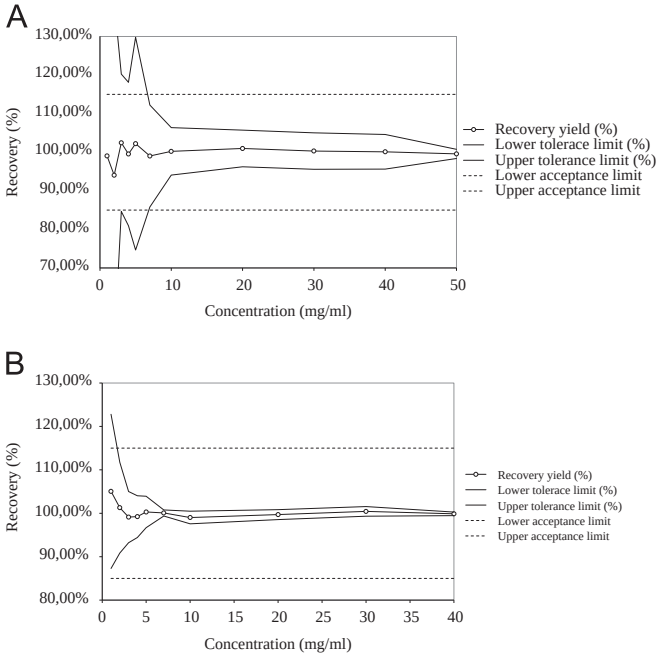

Fig. 4. Accuracy profile of PLS model of 5-fluorouracil (A) and gemcitabine (B). The dotted line is the mean recovery, the dashed lines are the β-expectation tolerance limits (β=95%) and the plain lines represent the acceptance limits (+/−15%).

glass vial with chloride sodium 0.9% as background reference. Negative values of spectral data correspond to a decrease of the water signal in comparison to the background. NIR spectra from 4000 to 13,000 cm^{-1} of 1 to 40 mg/ml GEM and 1 to 50 mg/ml 5FU solutions are shown on Fig. 2.

From 5755 to 4000 cm^{-1} and from 7193 to 6449 cm^{-1}, signal of spectra are unusable due high absorbance of O-H vibrational band of water. From 6449 to 5755 cm^{-1}, the data exploitation was very tenuous due to the low intensity of the light in this spectral zone.

On contrary, from 13,000 to 7193 cm^{-1}, numerous bands can be observed. A variation of signal intensity in accordance to concentration was observed for GEM samples such as bands at 8441 cm^{-1} and 8664 cm^{-1} (Fig. 2A). Whereas, the trend is not as soon as evident for 5FU samples spectra (Fig. 2B), a variation of the signal in accordance to concentration can also be observed. When the phenomenon of diffuse reflection is used for the near infrared measurement, e.g. in measurements on powders, it is very common to observe that the baselines of the spectra are offset. This kind of spectral behavior is unexpected when absorption spectra of liquids are recorded by transmission. However, phenomena of reflections on the glass surface and slight differences in thickness between the blank vial and the vial samples could explain the offsets observed in our measurements. These offsets are small compared to the amplitude signal in the case of gemcitabine, but can not be neglected in the case of 5FU. Preprocessing of spectra should then be considered: it is a usual operation in analyzing near infrared data. Fig. 2C shows the spectra of Fig. 2B after straight line subtraction preprocessing applied to construct the optimal calibration model.

3.1. Determination of calibration models

Regarding RMSECV and RMSEP of 0.483 mg/ml and 0.519 mg/ml respectively for 5FU and 0.139 mg/ml and 0.108 respectively for GEM (Fig. 3), 3 latent variables were chosen to PLS regression model used to predict 5FU but also GEM concentration values. For 5FU, two spectral ranges from 10,691.4 to 9920.0 and 9152.5 to 8377.3 cm^{-1} pre-treated by straight line subtraction (Fig. 2C) were used to build the optimal PLS predictive model characterized by a RMSEC of 0.472 mg/ml, a bias of 0.157 mg/ml and a R^2 of 0.9992. For GEM, two spectral ranges from 8948.1 to 8469.8 and 8404.3 to 8265.4 cm^{-1} without pre-treatment (Fig. 2A) were also used to predict the GEM value with a RMSEC of 0.129 mg/ml, a bias of 0.0342 mg/ml and a R^2 of 0.9999.

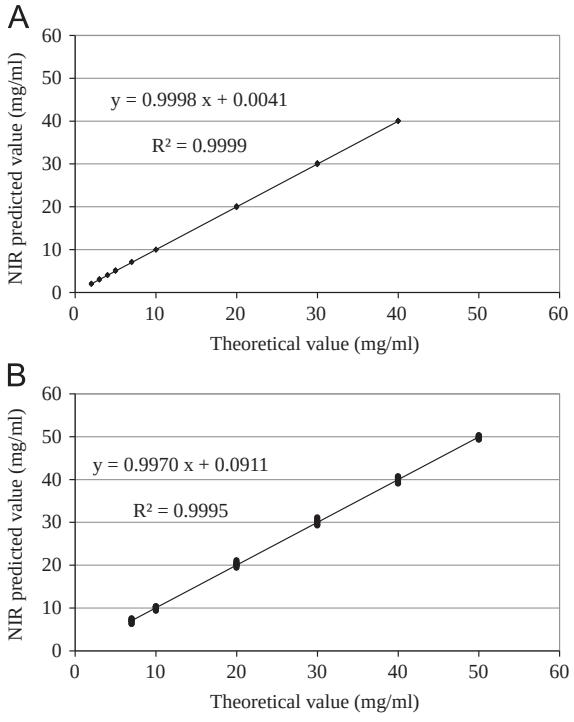

Fig. 5. Correlation diagram between NIR prediction and theoretical values for the validated calibration model for gemcitabine (A) and 5-fluorouracil (B).

In addition, other parameters such as the residual prediction deviation (RPD) can be calculated as a quality indicator of the models. RPD represent the ratio between the standard deviation of reference data in the prediction sample set (SD) and the standard error of prediction (SEP). Williams [21,22] suggested that R^2 value greater than 0.9 associated to RPD values greater than 3 indicate excellent quantitative information. In term of those outlined criteria, both models with R^2 greater than 0.9992 and RPD of 36 for 5FU and 125 for GEM displayed excellent prediction capacity.

3.2. Determination of calibration models performances

3.2.1. Accuracy profile

To evaluate performance of PLS predictive models, the accuracy profile was established on predicted concentration values of the calibration set. Limits of acceptance were set at +/−15% with a maximum risk at 5%. Calibration curves were prepared in the range of 1–50 mg/ml for 5FU and 1–40 mg/ml for GEM. No weighting was applied. The low limit of quantification (LLOQ) was graphically determined from the accuracy profile for the lowest concentration included in the limits of acceptance of +/−15% with a maximum risk of 5%. LLOQ were 7.0 mg/ml and 2.0 mg/ml for 5-fluorouracil and gemcitabine respectively (Fig. 4).

As a consequence, linear regression was recalculated regarding the limit of quantification. Thus, linear calibration were considered for concentration range from 7 to 50 mg/ml for 5-fluorouracil and from 2 to 40 mg/ml for gemcitabine with a coefficient correlation (R^2) higher than 0.9995 for those two molecules (Fig. 5).

The precision of the calibration method was determined across repeatability and intermediate precision using calibration set samples (Table 1). Trueness was expressed as the ratio between theoretical and the average measured concentration and was ranged from −0.98% to 0.97% for 5-fluorouracil and −0.98% to 1.28% for gemcitabine. Repeatability and intermediate precision were interpreted using relative standard deviation and did not exceed 5.07% and 6.93% respectively for 5-fluorouracil and 3.07% and 4.97% respectively for gemcitabine.

3.2.2. Specificity

For NIRS methods, PLS models are not enough sufficient to ensure discriminant identification. Specificity constitutes an essential requirement for analytical method validation. This parameter measures the ability of the method to identify the molecule of interest in the sample regarding a library of other cytotoxic molecules. More than 40 molecules can be used to treat cancer. However, this study is a pilot study to evaluate the potentiality of NIRS to identify and quantify active drug by non-invasive method, explaining that only GEM and 5FU have been studied. At present, NIR library only contained those two molecules but will be completed in the future by other active drugs. It must be noted that the spectra of GEM and 5FU are quite different in the spectral ranges involved in the calibrations models so that we can expect that the solutions of both molecules could be identified thanks to their infrared spectra. Actually, a cluster analysis of the spectra used in the calibration and validation sets demonstrated a separation in two groups for all solutions whose a concentration is higher than the detection limit of the quantification model.

3.3. Pharmaceutical preparation samples analysis

In accordance to Guidelines on the use of NIRS [20], the external validation set was independent to calibration and validation set with samples extracted from production. Thus, samples covered the full range of variations in the sample population. Because 5FU samples have theoretical concentration inferior to the LLOQ, those samples were not analyzed. On contrast, 40 GEM samples were analyzed by the routinely method used in our laboratory (FIA-UV) and compared to prediction value obtained by NIRS. The Bland Altman method was used to compare the two methods. 95% of the difference scores were contained into the limits of agreement ranging from −0.099 mg/ml to 0.345 mg/ml (Fig. 6). The bias of 0.123 mg/ml (95% CI bias from 0.087 to 0.159) shows a significant difference of measurements between the two methods. However, the Pearson coefficient of −0.09 (95% CI from −0.391 to 0.228) signed a correlation between the two methods. However, the Bland Altman plot shows a significant mean difference of the measurement between NIR and FIA UV methods of 0.123 mg/ml (95% CI bias from 0.087 to 0.159). However, this bias is independent of the concentration and can be due to the use of different calibration set data to predict concentration value by FIA UV. However, the maximal error of 8.35% observed for a mean concentration of 4.088 mg/ml (0.341 mg/ml of difference) was

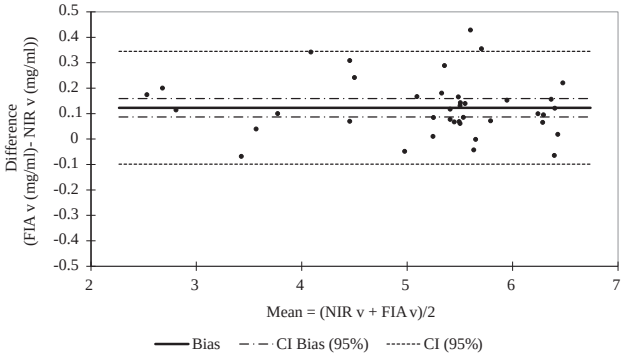

Fig. 6. Bland–Altman plot of the difference between near infrared predicted value (NIRv) and flow injection analysis value (FIAv) is drawn against the mean of NIRv and FIAv in the 40 paired measurements in the study extracted from cytotoxic drugs preparation before patient administration.

Table 1
Trueness, repeatability and intermediate precision for gemcitabine and 5-fluorouracil

Theoretical concentration (mg/ml)	Gemcitabine			5-fluorouracil		
	Trueness (%)	Repeatability (%)	Intermediate precision (%)	Trueness (%)	Repeatability (%)	Intermediate precision (%)
2	1.28	3.07	4.97	–	–	–
3	−0.88	1.40	2.70	–	–	–
4	−0.77	1.94	2.55	–	–	–
5	0.30	0.98	1.70	–	–	–
7	0.08	0.38	0.40	−0.98	5.07	6.93
10	−0.98	0.40	0.69	0.22	3.65	3.65
20	−0.31	0.39	0.58	0.97	2.30	2.64
30	0.44	0.62	0.65	0.30	1.27	2.22
40	−0.12	0.25	0.25	0.11	0.59	1.81
50	–	–	–	−0.43	0.71	0.71

inferior to the 15% of specification limits available at hospital for the control of pharmaceutical cytotoxic drugs preparations. Whereas, FIA UV method is more sensitive for low concentrations than NIR spectroscopy, the results of this study show a good prediction ability of NIR spectroscopy to predict 5FU and GEM concentrations directly through a glass packaging. This method represents an interesting alternative. In fact, different types of preparations are used to administrate cytotoxic drugs but some of them could not be controlled by the FIA UV routinely method. Syringes are incompatible with the sample volume of around 1 ml required and elastomeric infusion pumps used have not got any sampling site. Those two types of pharmaceutical preparations are generally used to administrate high concentrations of cytotoxic drug. Regarding the linear range and the possibility of direct measurement, NIRS should be explored as an interesting alternative of analytical control. Moreover, NIRS as well as FIA UV has a run time is inferior to 1 min allowing the use of NIRS for analytical control at the end of cytotoxic preparation process without delayed liberation.

4. Conclusion

This study demonstrated the ability of NIR spectroscopy coupled with PLS regression to predict cytotoxic drugs concentrations in aqueous solution. Moreover, regarding the capacity of direct measurement through the packaging, NIRS contributes to improve the safety of the patient in addition to staff protection by limiting cytotoxic drug handling. As a non-invasive, non-destructive but also rapid analytical method, NIRS should be extended to other cytotoxic drugs to an exhaustive and more safety control of cytotoxic drugs preparations.

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