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Ultra-sensitive LC–MS/MS method for the quantification of gemcitabine and its metabolite 2′,2′-difluorodeoxyuridine in human plasma for a microdose clinical trial

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ABSTRACT

In microdose clinical trials a maximum of 100 μg of drug substance is administered to participants, in order to determine the pharmacokinetic properties of the agents. Measuring low plasma concentrations after administration of a microdose is challenging and requires the use of ulta-sensitive equipment. Novel liquid chromatography-mass spectrometry (LC–MS/MS) platforms can be used for quantification of low drug plasma levels. Here we describe the development and validation of an LC–MS/MS method for quantification of gemcitabine and its metabolite 2′,2′-difluorodeoxyuridine (dFdU) in the low picogram per milliliter range to support a microdose trial. The validated assay ranges from 2.5–500 pg/mL for gemcitabine and 250–50,000 pg/mL for dFdU were linear, with a correlation coefficient (r^2) of 0.996 or better. Sample preparation with solid phase extraction provided a good and reproducible recovery. All results were within the acceptance criteria of the latest US FDA guidance and EMA guidelines. In addition, the method was successfully applied to measure plasma concentrations of gemcitabine in a patient after administration of a microdose of gemcitabine.

1. Introduction

Microdose studies are exploratory investigational new drug (eIND) trials that can be conducted in a phase 0 context. The aim of such trials is to accelerate drug development by early selection of promising candidates. A microdose is defined as 1/100th of the therapeutic dose or the dose calculated to yield a pharmacological effect, with a maximum dose of 100 μg [1,2]. As no clinical effect is expected after administration of such a low dose, microdoses are considered harmless.

After administration of a microdose, pharmacokinetic data of the investigated drug are acquired and evaluated. Early establishment of such parameters might shorten the overall development time and increase success rates of drug approval.

Administration of a microdose results in low systemic plasma concentrations. Determining such low concentrations requires the use of sensitive analytical techniques. Commonly used analytical tools in these cases are accelerator mass spectrometry (AMS) and liquid chromatography-mass spectrometry (LC–MS/MS) [3]. Although AMS is known for its high sensitivity and specificity, the low availability and the use of radiolabeled drugs makes this technique expensive. Therefore, the new generation of ultra-sensitive LC–MS/MS provides a good alternative with measurements that have reached the picogram per milliliter level without using radioactive labeled drugs.

An LC–MS/MS method was developed and validated for simultaneous quantification of gemcitabine (dFdC) and its metabolite 2′,2′-difluorodeoxyuridine (dFdU) to support a microdose trial. Gemcitabine is a nucleoside analog that can be prescribed for treatment of several cancer types. The main antitumor effect is caused by

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its triphosphate metabolite dFdCTP, inhibiting DNA synthesis after being incorporated into the DNA [4]. Previously published methods for the quantification of gemcitabine and dFdU have insufficient sensitivity to be applied in a microdose trial. The lower limit of quantification (LLOQ) in these assays ranges from 0.25–125 ng/mL and 1–1250 ng/mL for gemcitabine and dFdU, respectively [5–11]. We developed a method with 100-fold increased sensitivity to enable analysis of patient samples with low picogram per milliliter concentrations. The focus of this paper is on the development and validation of such a highly sensitive LC–MS/MS method by preserving accurate and precise measurements.

2. Materials and methods

2.1. Chemicals

Gemcitabine hydrochloride (dFdC HCl), 2′,2′-difluorodeoxyuridine, $^{13}C,^{15}N_2$-gemcitabine hydrochloride and $^{13}C,^{15}N_2$-2′,2′-difluorodeoxyuridine were purchased from Alsachim (Illkirch Graffenstaden, France). Acetonitrile, methanol and water (all Supra-Gradient grade) were from Biosolve Ltd (Valkenswaard, The Netherlands). Ammonium acetate (98%) and tetrahydrouridine were supplied from Merck (Amsterdam, the Netherlands). Water (distilled) used for sample preparation came from B. Braun Medical (Melsungen, Germany). Blank human dipotassium ethylenediaminetetraacetic acid (K_2EDTA) plasma was obtained from the department of clinical chemistry (MC Slotervaart, the Netherlands).

2.2. THU stabilized plasma

In human plasma, gemcitabine is deaminated by cytidine deaminase leading to the formation of dFdU. Tetrahydrouridine (THU) is a potent inhibitor of cytidine deaminase and can be added to plasma to prevent deamination. THU was dissolved in water to obtain a 10 mg/mL solution. Consequently, control human K_2EDTA plasma was spiked with this solution at a final concentration of 0.1 mg/mL. This THU stabilized control human K_2EDTA plasma was used for making working solutions, calibration standards and quality control (QC) samples.

2.3. Stock solutions and working solutions

Separate stock solutions of 1 mg/mL for calibration standards and QC samples were prepared in water for each analyte (corrected for potency). The stock solutions were further diluted with THU stabilized control K_2EDTA plasma to obtain separate working solutions. Stock solutions of the internal standards (IS) were also prepared at 1 mg/mL in water. A mixture of internal standard stock solutions was prepared and diluted with water to obtain a working solution IS (WIS) that was used for sample pretreatment. This WIS contained 10 ng/mL $^{13}C,^{15}N_2$-gemcitabine and 100 ng/mL $^{13}C,^{15}N_2$-2′,2′-dFdU. Stock solutions and working solutions were stored at −20 °C.

2.4. Calibration standards, quality control samples

Calibration samples were prepared freshly prior to each validation run, by spiking 25 μL working solution to 475 μL THU stabilized control K_2EDTA plasma. QC samples were prepared in batches and stored at −20 °C. Eight calibration standards were used in this assay and a limit of detection (LOD) was added to determine the lowest analyte concentration to be reliably distinguished from the noise. Concentrations of the calibration standards were 5, 10, 25, 50, 100, 250, 400 and 500 pg/mL with an LOD of 2.5 pg/mL for gemcitabine and 500, 1000, 2500, 5000, 10,000, 25,000, 40,000, 50,000 pg/mL with an LOD of 250 pg/mL for dFdU. Quality control samples were prepared at concentrations 5, 15, 50 and 375 for gemcitabine and 500, 1500, 5000, 37,500 for dFdU.

2.5. Sample preparation

Samples were thawed prior to processing and 200 μL was aliquoted in 1.5 mL containers. Each sample was spiked with 20 μL WIS, except for double blank calibration samples. Samples were prepared with solid phase extraction (SPE) using Oasis HLB 1cc vac cartridges (Waters, Milford, MA, USA). The cartridges were first equilibrated with 0.5 mL methanol and 0.5 mL water, respectively. After equilibration, plasma samples were transferred to the cartridges and washed with 0.5 mL water. The cartridges were dried under a maximal vacuum for 10 min and samples were eluted with 0.4 mL methanol. Afterwards, the samples were dried under a gentle stream of nitrogen at 40 °C and the dried extract was subsequently reconstituted with 80 μL of reconstitution solvent (10 mM ammonium acetate in water-acetonitrile (93:7, v/v)) by vortex mixing and shaking (10 min at 1250 rpm). The final extracts were transferred to autosampler vials with insert.

2.6. LC equipment and conditions

Gemcitabine and dFdU were chromatographically separated using a Shimadzu LC system with a binary pump, a degasser, an autosampler and a valco valve (Nexera 2 series, Shimadzu corporation, Kyoto, Japan). The autosampler temperature was kept at 4 °C and the column oven at 30 °C. Mobile phase A consisted of 10 mM ammonium acetate in water-acetonitrile (93:7, v/v) and mobile phase B of 100% acetonitrile. Gradient elution was applied at a flow rate of 0.2 mL/min through a Acquity UPLC HSS T3 column (100 Å, 2.1 × 150 mm, 1.8 μm) with an additional Acquity UPLC HSS T3 Vanguard pre-column (100 Å, 2.1 × 5.0 mm, 1.8 μm) (Waters, Milford, MA, USA). The following gradient was applied: 0% B (0.0–7.0 min), 0–80% B (7.0–7.5 min), 80% B (7.5–10 min), 0% B (10–13 min). The divert valve directed the flow to the mass spectrometer between 2.0 and 7.0 min and the remainder to the waste container.

2.7. MS equipment and conditions

A triple quadropole mass spectrometer 6500 (Sciex, Framingham, MA, USA) with a turbo ion spray (TIS) interface operating in the positive mode was used as a detector. For quantification, multiple reaction monitoring (MRM) chromatograms were acquired and processed using Analyst® 1.6.2 software (AB Sciex). General and analyte specific mass spectrometric parameters are listed in Table 1 and the structures and the proposed fragmentation patterns of the analytes are depicted in Fig. 1.

2.8. Validation procedures

A full validation of the assay was performed based on the United States Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines for bioanalytical method validation [12,13]. The following aspects were established during the validation: calibration model, accuracy, precision, carry-over, selectivity (endogenous and cross analyte/IS interferences), matrix effect, recovery, dilution integrity and stability.

2.8.1. Calibration model

Eight non-zero calibration standards were prepared freshly in duplicate for each run and were analyzed in three separate runs. Calibration linearity was determined by plotting the peak area ratio of the analyte/IS against the corresponding concentration (x)

Table 1
Above: General mass spectrometric parameters. Below: Analyte specific mass spectrometric parameters for gemcitabine, 2′,2′-difluorodeoxyuridine (dFdU) and the internal standards. Abbreviations: min = min, V = voltage, au = arbitrary units, ms = milliseconds, *m/z* = mass-to-charge ratio.

General settings	
Run duration	13 min
Ionspray voltage	3000 V
Nebulizer gas	40 au
Turbo gas/heater gas	70 au
Curtain gas	35 au
Collision gas	10 au
Temperature	450 °C
Dwell time	150 ms

	Gemcitabine	Gemcitabine IS	dFdU	dFdU IS
Parent mass	264 *m/z*	267 *m/z*	265 *m/z*	268 *m/z*
Product mass	112 *m/z*	115 *m/z*	113 *m/z*	116 *m/z*
Collision energy	21	21	21	21
Collision exit potential	14	14	14	14
Declustering potential	51	51	71	71
Retention time	3.42	3.42	5.35	5.35

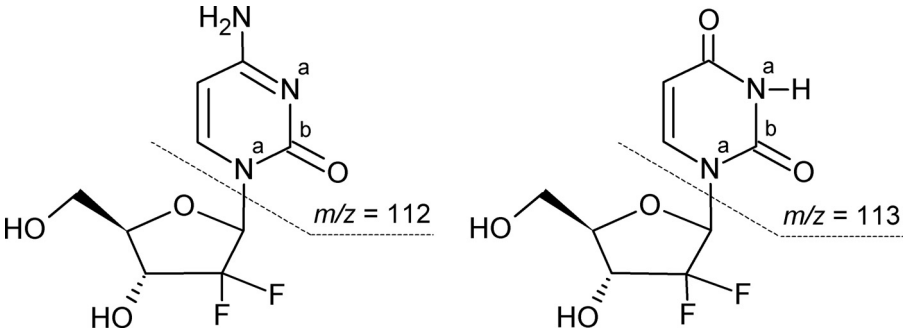

Fig. 1. Chemical structures of gemcitabine and dFdU, including the proposed *m/z* fragments. The stable isotopes in the internal standards are indicated with [a] (^{15}N) and [b] (^{13}C).

of the calibration standard. For all analytes, the reciprocal of the squared concentrations ($1/x^2$) was used as a weighting factor. Deviations from the mean calculated concentrations should be within 85–115% of the nominal concentrations in at least 75% of non-zero calibration standards. At the LLOQ, a deviation of 20% was permitted.

2.8.2. Limit of detection

A limit of detection was included to determine the lowest analyte concentration to be reliably distinguished from the noise. The signal-to-noise ratio of the LOD should be ≥3

2.8.3. Accuracy and precision

Intra- and inter-assay accuracies and precisions of the method were determined by analyzing five replicate QC samples in three consecutive runs at LLOQ, low, mid and high concentration levels. Accuracy was expressed as the bias and precision was calculated as the coefficient of variance (CV) according to the following equations [14]:

$$\text{Intra} - \text{assay bias}\,(\%) = 100\% \cdot (\textit{mean measured conc.per run} - \textit{nominal conc.}) / (\textit{nomimal conc.})$$

$$\text{Inter} - \text{assay bias}\,(\%) = 100\% \cdot (\text{overall mean measured conc.} - \text{nominal conc.}) / (\text{nominal conc.})$$

$$\text{Intra} - \text{assay CV}\,(\%) = 100\%\,(\textit{SD of the measured conc.per run}) / (\textit{mean measured conc.per run})$$

$$\text{Inter} - \text{assay CV}(\%) = \frac{\sqrt{\left(\frac{s^2_{overall}/\left((n_1+\ldots+n_a-1)-\left((n_1-1)s_1^2+\ldots+\left((n_a-1)s_a^2\right)\right)\right)}{a-1}\right) - \left(\frac{(n_1-1)+\ldots+(n_a-1)s_a^2}{n_1+\ldots+n_a-a}\right)}}{\left(\frac{n}{a}\right)}$$

The acceptance criteria for both parameters were ±15% for QC low, QC mid and QC high and ±20% for QC LLOQ.

2.8.4. Carry-over

Carry-over was investigated by injecting two double blank samples after a calibration standard with the highest concentration (upper limit of quantification, ULOQ). The peak areas in the blank processed samples were expected to be <20% of the peak area in the LLOQ sample.

2.8.5. Selectivity

Six individual batches of THU stabilized control K_2EDTA plasma were used to assess the specificity and selectivity of the method. A double blank sample and a sample spiked at the LLOQ were processed of each batch. The areas of co-eluting peaks in the double blank samples should be <20% of the peak area in LLOQ samples in each batch. Furthermore, the areas of peaks in the double blanks co-eluting with internal standards were expected to be <5% of the

peak area of the mean internal standard response. For LLOQ samples, sample inaccuracies were expected to be within 80–120% of the nominal concentration in at least 5 out of 6 samples. Cross-analyte and IS interference was determined with single samples that were separately spiked with one analyte or IS at ULOQ concentration or nominal concentration, respectively. To ensure that compounds did not interfere with the quantification of other analytes, the cross-analyte/IS interference should be ≤20% of the peak area in LLOQ samples and ≤5% for the IS.

2.8.6. Matrix effect and recovery

The matrix effect was determined in six different batches of THU stabilized control human plasma at low and high concentration levels in singular. The following samples were prepared: QC samples in the presence of matrix (each lot of blank sample processed to dried extract, spiked with an academic solution at low and high concentrations) and QC samples in the absence of matrix (a pure solution of the analyte at low and high concentrations). The matrix factor was calculated for each batch by calculating the ratio of the peak area in the presence of matrix to the peak area in absence of matrix. Additionally, the IS-normalised matrix factor was calculated by dividing the matrix factor of the analyte by the matrix factor of the IS.

The recovery was investigated at low and high concentration levels (n = 3) in one batch of THU stabilized control human plasma. The recovery was determined by calculating the ratio of the peak area of processed QC samples to the peak area in presence of matrix (blank sample processed to dried extract, spiked with an academic solution at low and high concentrations). The coefficient of variance (CV) for the matrix factor and the recovery should be <15%. [12]

2.8.7. Dilution integrity

Dilution integrity was investigated with five replicate plasma samples with a concentration above the ULOQ. These samples were diluted 10-fold by adding 30 μL sample to 270 μL THU stabilized control human plasma. An accuracy of within −15% and +15% of the nominal concentration was acceptable.

2.8.8. Stability

Short-term stability experiments were performed in plasma after storage at room temperature (20–25 °C) and at −20 °C. Further stability assessments were done in dried extracts and final extracts at 4 °C. The effect of 3 freeze (−20 °C)/thaw cycles on the stability of each compound was investigated after thawing samples to room temperature with a minimum interval of 12 h on 3 separate occasions and comparison with freshly prepared calibration samples. Short-term stability in plasma was determined after 1 month and long-term stability will be investigated after 3 months, 6 months and 1 year of storage at −70 °C. Above described stability experiments were performed in triplicate at low and high concentration levels. Analytes were considered stable under specific conditions when 85–115% of the initial concentration were recovered. Short-term stock stability of one month was previously established [5]. The results of 26-month stock stability at −20 °C are described in this manuscript. Analytes are considered stable in the stock solution when 95%–105% of the original concentration is recovered.

2.9. Clinical application

This analytical assay was used for sample analysis in a microdose trial with gemcitabine. The study was conducted in accordance with the International Conference on Harmonisation guidelines for Good Clinical Practice and the Declaration of Helsinki. The protocol was approved by the Independent Ethics Committee of the Netherlands Cancer Institute. Patients with advanced cancer received 100 μg of gemcitabine via a 30-min infusion. Blood was drawn at the following time points: t = 0 (predose), t = 15 min (1/2th of infusion), t = 30 min (end of infusion), t = 45 min, t = 60 min, t = 75 min, t = 90 min, t = 105 min, t = 2 h, t = 4 h and t = 8 h. Blood was collected in 4 mL tubes pre-spiked with 40 μL THU to obtain a final concentration of 0.1 mg/mL. Whole venous blood was centrifuged for 10 min at 2000 g at 4 °C and the plasma was transferred to 2.0 mL containers. Samples were stored at −70 °C directly after processing. Afterwards, the patients went off study and were treated in their best interest with standard of care gemcitabine.

3. Results and discussion

3.1. Development

3.1.1. Sample preparation

Several conventional sample preparation methods were considered during method development, such as protein precipitation and solid phase extraction (SPE). Protein precipitation showed a low recovery and high background noise, while SPE efficiently removed interferences and thereby produced well purified final extracts. To meet sensitivity requirements, SPE was chosen as the method for sample preparation. Oasis® MCX, MAX and HLB SPE cartridge sorbents were evaluated during method development. Since gemcitabine and dFdU are polar compounds, SPE with Oasis® HLB cartridges gave the highest recovery compared to other cartridges.

During the development of this assay, K_2EDTA plasma from Bioreclamations LLC (Hicksville, NY, USA) was used for the preparation of calibration standards and QC samples. Double blank samples of this batch showed an interference at the retention time of gemcitabine, which interfered with low calibration standards. To further develop this method, a new batch of plasma was collected at the department of clinical chemistry (MC Slotervaart, the Netherlands) that did not contain this interference. This batch was used for development and validation of the assay. During the microdose trial, pre-dose samples were collected. These samples did not contain the described interference.

3.1.2. Mass spectrometry and chromatography

Previously published methods for the quantification of gemcitabine and dFdU required improvements regarding sensitivity to measure low plasma concentrations. Use of an ultra-sensitive MS system (i.e. QTRAP6500) already improved selectivity, but optimization of the assay was necessary to reach a sufficient LLOQ. Therefore, we used an UPLC column to improve separation of the compounds and to improve peak shape. Since using a UPLC column clearly increased resolution and the shape of eluting peaks, a nano-LC system with similar column material was tested to further enhance peak separation and signal to noise ratio. Implementation of this nano-LC system, however, did not further improve the sensitivity of the assay. Thus, an UPLC system was used to develop and to validate this method. Representative chromatograms of a blank sample, QC LLOQ and a patient sample at t = 8 h are presented in Fig. 2 for both analytes. Gemcitabine and dFdU were baseline separated. The first peak in the gemcitabine chromatogram corresponds to THU at the transition of gemcitabine (m/z 164 → 111.9), as non THU stabilized control plasma does not show this peak.

3.2. Validation procedures

3.2.1. Calibration model

All the calibration curves were constructed using a weighting factor of $1/x^2$ and were fitted linearly with a minimum correlation coefficient (r^2) of 0.9964 for gemcitabine and 0.9962 for dFdU. The assay was linear for the validated concentration ranges of 5–500 pg/mL for gemcitabine and 500–50,000 pg/mL for dFdU.

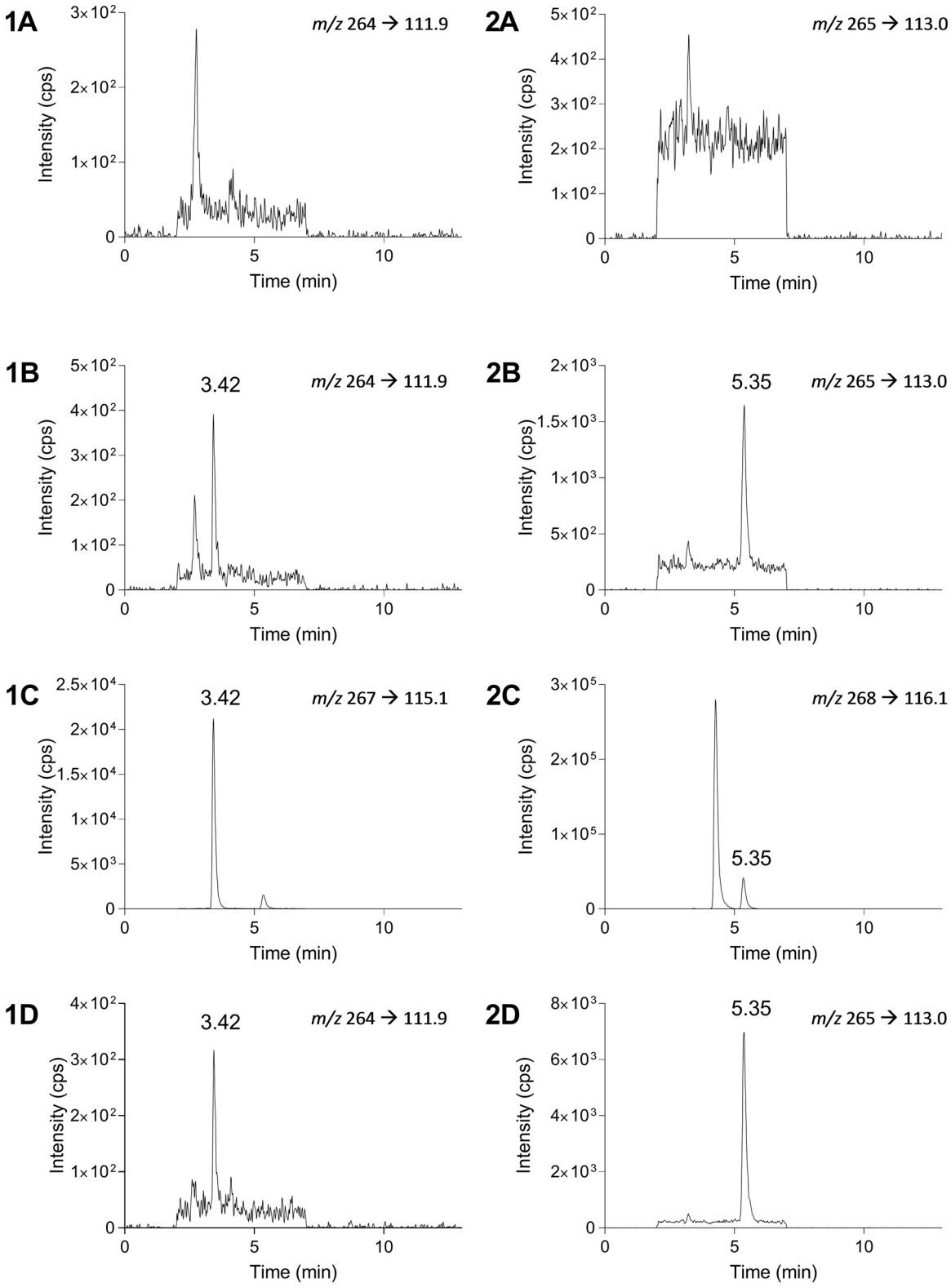

Fig 2. Representative LC–MS/MS chromatograms of a blank sample (1A and 2A), gemcitabine LLOQ (1B), dFdU LLOQ (2B), gemcitabine internal standard (1C), dFdU internal standard (2C), gemcitabine and dFdU in a patient sample at t = 8 h (1D and 2D). The second peak in 1C at 5.35 min belongs to the isotopically labeled dFdU, and the first peak in 2C at 4.27 min is an unknown endogenous interference.

3.2.2. Limit of detection

All LOD samples had a single-to-noise ratio of at least 3. To determine plasma concentrations between the LOD and the LLOQ, the LOD could be included in the calibration range. The calibration model (linear fit with a weighting factor of $1/x^2$) remained the same and all calibration samples and QC samples remained within the acceptance criteria. In the application phase of the method it is feasible to include the LOD in the calibration model to quantify plasma concentrations between the LLOQ and the LOD as long as the signal-to-noise ratio is at least 3. In pharmacokinetic analysis,

Table 2
Assay performance data for gemcitabine and its metabolite 2′,2′-difluorodeoxyuridine (dFdU).

Analyte	Nominal conc. (pg/mL)	Intra-assay (n = 15)		Inter-assay (n = 15)	
		Bias (%)	C.V. (%)	Bias (%)	C.V. (%)
Gemcitabine	5	6.4–11.5	2.8–6.2	8.9	1.3
	15	−2.5–4.7	4.8–7.2	1.0	2.3
	50	2.9–5.5	3.8–6.0	4.5	*
	375	2.5–10.6	2.2–13.2	7.4	2.2
dFdU	500	2.1–11.6	5.3–8.0	8.0	3.7
	1500	2.3–3.7	4.1–8.5	3.2	*
	5000	−0.6–4.7	2.6–6.2	2.4	1.8
	37500	6.7–11.9	1.2–3.3	9.9	2.4

* The inter-assay precision could not be calculated because there is no significant additional variation due to the performance of the assay in different batches. Abbreviations: conc. = concentration, C.V. = coefficient of variation.

incorporation of the concentrations between the LLOQ and LOD show superior pharmacokinetic models in terms of bias and precision compared to models that exclude these concentration data [15].Accuracy and precision

Assay performance data of gemcitabine and dFdU are presented in Table 2. Inter-assay accuracy, intra-assay accuracy and the precision were ≤15% for low, mid and high concentrations and ≤20% for the LLOQ concentrations. Therefore, accuracy and precision were within the acceptance criteria.

3.2.3. Specificity and selectivity

MRM chromatograms of six batches of control human plasma contained no co-eluting peaks larger than 20% of the area at the LLOQ level of the analytes and no co-eluting peaks larger than 5% of the area of both internal standards. The influence of different batches control human plasma on the accuracy and precision at LLOQ level was investigated. The accuracies at LLOQ level were in all six batches of control human plasma within 20% of the nominal concentration. Cross-interference of co-eluting peaks in separately spiked samples were <20% of the QC LLOQ samples and thus within the required limits. For the internal standards, the interference was less than 5% and thus also within the acceptance criteria.

3.2.4. Dilution integrity

The concentrations of 10-fold diluted samples were within 85–115% of the nominal concentration in five replicates. Intra-assay bias and intra-assay variability were 2.2% and 5.5% for gemcitabine and 3.6% and 2.9% for dFdU, respectively. These results show that samples with concentrations >ULOQ can be diluted up to 10-fold.

3.2.5. Carry-over

There were no peaks observed in the first blank processed sample, which means that there was no carry-over for gemcitabine, dFdU or the internal standards.

3.2.6. Matrix factor and recovery

The CV for the IS-normalised matrix factor was below 15% at the tested concentrations for each compound. The total recovery was determined at two concentration levels and was >64.8% for gemcitabine, >64.5% for dFdU, >70.7% for $^{13}C,^{15}N_2$-gemcitabine and >82.6% for $^{13}C,^{15}N_2$-dFdU. Although the recovery of the analytes was below 65%, the method was sufficient to reach an LOD of 2.5 pg/mL and 250 pg/mL for gemcitabine and dFdU, respectively. The coefficient of variance did not exceed 15%.

3.2.7. Stability

The results of the investigated stability are presented in Table 3. Gemcitabine and dFdU are stable in stock solution for 26 months. All other experiments demonstrate adequate stability of both analytes in biomatrix, dried extract and final extract. Long-term stability assessment in THU stabilized control human plasma was measured up to 1 month and is still ongoing.

4. Clinical application

The validated gemcitabine assay is used to support a clinical microdose trial. A plasma concentration-time profile of gemcitabine after administration of a microdose is depicted in Fig. 3. The patient received a single 100 μg dose of gemcitabine via a 30-min infusion. This dose is at least 10,000-fold lower than a therapeutic gemcitabine dose of 1000–1250 mg/m^2. All measurements were within the validated range after 4-fold dilution of plasma samples at 1/2th (t = 15 min) of infusion and end of infusion (t = 30 min). The LLOQ was sufficiently low to measure gemcitabine and dFdU

Table 3
Stability parameters for gemcitabine and 2′,2′-difluorodeoxyuridine (dFdU).

Analyte	Conditions	Matrix	Nominal concentration (pg/mL)	Measured concentration (pg/mL)	Bias (%)	C.V. (%)	n
Gemcitabine	−20 °C, 26 m	Water (stock)	1.05 ng/mL	1.01 ng/mL	−0.44	0.63	3
	3 F/T (20 °C/RT)	Plasma	15	16.2	8.9	1.9	3
			375	391	3.2	6.0	
	RT, 3 d	Plasma	15	13.9	−6.9	1.8	3
			375	416	10	1.8	
	−70 °C, 1 m	Plasma	15	16.2	8.0	5.7	3
			375	360	−5.0	3.0	
	2–8 °C, 4 d	Dried extract	15	15.4	2.9	3.7	3
			375	415	10	2.5	
	2–8 °C, 7 d	Final extract	15	14.5	−2.0	3.1	3
			375	409	8.0	3.2	
dFdU	−20 °C, 26 m	Water (stock)	1.03 ng/mL	1.03 ng/mL	0.06	3.2	3
	3 F/T (20 °C/RT)	Plasma	1500	1443	−2.4	6.3	3
			37500	39900	6.4	3.8	
	RT, 3 d	Plasma	1500	1437	−2.7	0.7	3
			37500	42233	11	2.4	
	−70 °C, 1 m	Plasma	1500	1600	6.7	2.3	3
			37500	39033	4.1	1.5	
	2–8 °C, 4 d	Dried extract	1500	1613	7.6	0.9	3
			37500	41967	12	1.7	
	2–8 °C, 7 d	Final extract	1500	1640	11	2.6	3
			37500	41733	10	1.0	

Abbreviations: C.V. = coefficient of variation, m = months, d = days, RT = room temperature, F/T = freeze/thaw.

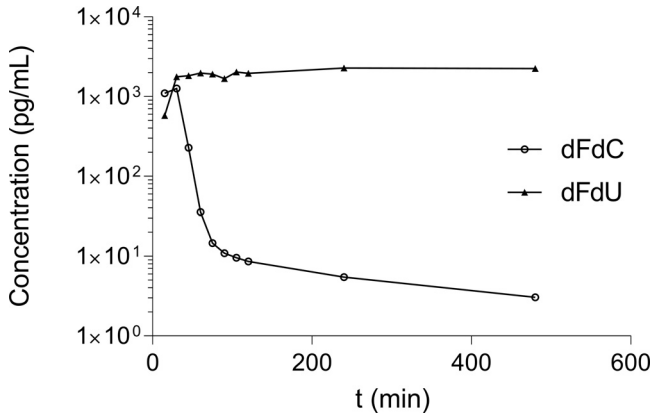

Fig. 3. Plasma concentration-time profile of gemcitabine (dFdC) and 2′,2′-difluorodeoxyuridine (dFdU) following an intravenous dose of 100 μg gemcitabine in a patient with non-small cell lung cancer via a 30-min infusion.

up to eight hours after start of the infusion with a plasma concentration of a 3 pg/mL at the final time point. From this plasma concentration-time profile, we can deduce pharmacokinetic data. These results demonstrate the applicability of this method for clinical gemcitabine microdose studies.

5. Conclusion

A ultra-sensitive LC–MS/MS method was developed and validated for the quantification of gemcitabine and its metabolite dFdU. The validated range is from 2.5–500 pg/mL and 250–50,000 pg/mL for gemcitabine and dFdU, respectively. The assay shows great improvement regarding sensitivity compared to previously published methods, while preserving the accuracy and precision. The method has been successfully implemented to support a microdose trial with gemcitabine.

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Conflicts of interest

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