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LC-MS/MS assay for the quantitation of FdCyd and its metabolites FdUrd and FU in human plasma

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Abstract

The hypomethylating agent 5-fluoro-2'-deoxycytidine (FdCyd, NSC 48006) is being evaluated clinically both via the intravenous route and via the oral route in combination with 3,4,5,6-tetrahydrouridine (THU), a potent inhibitor of FdCyd catabolism. To determine the pharmacokinetics of FdCyd and downstream metabolites, we developed and validated an LC-MS/MS assay for the quantitation of FdCyd, 5-fluoro-2'-deoxyuridine (FdUrd), and 5-fluorouracil (FU) in 0.2 mL human plasma. After acetonitrile protein precipitation, the sample was split and separate chromatography was achieved for FdCyd with a Synergi Polar-RP column and for FdUrd and FU with a Shodex Asahipak NH2P-50 2D column. Gradients of 0.1% acetic acid in acetonitrile and water were used. Detection with a Quattro micro quadrupole mass spectrometer with electrospray ionization in positive-ion (FdCyd) or negative-ion (FdUrd and FU) multiple reaction monitoring (MRM) mode. The assay was linear from 5–3000 ng/mL for all three analytes and proved to be accurate (96.7–105.5%) and precise (<8.1%CV), and fulfilled FDA criteria for bioanalytical method validation. We demonstrated the suitability of this assay for measuring FdCyd and metabolites FdUrd and FU in plasma from a patient who was administered 120 mg PO FdCyd 30 min after 3000 mg THU. Our LC-MS/MS assay will be an essential tool to further define the pharmacology of FdCyd in ongoing and future studies.

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Graphical abstract

Keywords

FdCyd; tandem mass spectrometry; metabolites; assay; validation

1 Introduction

Epigenetics research has furthered our understanding of malignancy and has provided novel targets for the development of cancer therapy [1–4]. Azacytidine and decitabine target DNA methyltransferases, the enzymes responsible for promoter hypermethylation [1, 5]. However, their poor aqueous stability limits their administration to parenteral dosing. 5-fluoro-2'-deoxycytidine (FdCyd, NSC 48006) is a DNA methylation inhibitor that is stable in aqueous solutions and has entered clinical testing [6]. Though chemically stable, in vivo, FdCyd is rapidly converted by cytidine deaminase (CD) to 5-fluoro-2'-deoxyuridine (FdUrd), followed by metabolism to 5-fluorouracil (FU). The metabolites have no hypomethylating effect, and their cytotoxicity may even prevent the incorporation of FdCyd into DNA, and thereby limit its hypomethylating effect [7]. To limit the catabolism of FdCyd to unwanted metabolites, FdCyd has been co-administered with 3,4,5,6-tetrahydrouridine (THU), a potent CD inhibitor [6–9]. The combination of IV FdCyd and IV THU has been investigated in a phase I study [6], and animal studies have been performed to support administration via the PO route [8, 10]. Previously published pharmacokinetic data [6, 8, 10, 11] were generated with an assay that utilized 2 serial HILIC columns for a run-time of approximately 1 hour, which proved to be unstable. In order to support ongoing clinical development of the PO combination of FdCyd and THU ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01534598) Identifier: NCT01534598), we developed and fully validated an assay for the quantitation of FdCyd, FdUrd, and FU in human plasma.

2 Experimental

2.1 Chemicals and reagents

FdCyd, FdUrd, and FU were obtained from the Developmental Therapeutics Program at the National Cancer Institute (Bethesda, MD, USA). [$^{15}\text{N}_2$, ^{13}C]-dFdC and [$^{15}\text{N}_2$]-FU were purchased from CDN Isotopes (Quebec, Canada). [$^{15}\text{N}_2$, ^{13}C]-FdUrd was purchased from

Toronto Research Chemicals, Inc. (Ontario, Canada). Acetonitrile (HPLC grade), water (HPLC grade), glacial acetic acid were purchased from Fisher Scientific (Fairlawn, NJ, USA). Control human plasma was purchased from Lampire Biological Laboratories (Pipersville, PA, USA). Nitrogen for evaporation of samples was purchased from Valley National Gases, Inc. (Pittsburgh, PA, USA). Nitrogen for mass spectrometrical applications was purified with a Parker Balston Nitrogen Generator (Parker Balston, Haverhill, MA, USA).

2.2 Chromatography

The LC system consisted of an Agilent (Palo Alto, CA, USA) 1100 autosampler and 1100 binary pump, a Phenomenex (Torrance, CA USA) Synergi Polar-RP (4 μ m, 2 $\times$ 100 mm) column for FdCyd and a Phenomenex Shodex Asahipak NH2P-50 2D (5 μ m, 2 $\times$ 150 mm) column for FdUrd and FU, and a gradient mobile phase. Mobile phase solvent A was 0.1% (v/v) acetic acid in acetonitrile, and mobile phase solvent B was 0.1 % (v/v) acetic acid in water.

For the FdCyd assay, the initial mobile phase composition was 100% solvent B pumped at 0.3 mL/min. Solvent B was decreased linearly over 1 min to 20%, At 4.1 minutes, the mobile phase was returned to 100% solvent B and the flow rate was increased to 0.5 mL/min. These conditions were maintained until 8 minutes. The total run time was 8 min.

For the FdUrd-FU assay, the initial mobile phase composition was 97% solvent A pumped at 0.3 mL/min for 3 min. Between 3 and 8 min, the percentage of solvent A was decreased linearly to 88%, while maintaining 0.3 mL/min flow rate. At 8.1 min, solvent A was decreased to 50% and the flow was increased to 0.7 mL/min. Between 10 and 10.1 min, the percentage of solvent A was returned to 97% and the flow rate was decreased to 0.6 mL/min. These conditions were maintained until 15 min. The total run time was 15 min.

2.3 Mass spectrometry

Mass spectrometric detection was carried out using a Waters (Milford, MA, USA) Quattromicro triple-stage, benchtop quadrupole mass spectrometer with electrospray ionization in positive-ion, multiple reaction monitoring (MRM) mode for FdCyd and in negative-ion, MRM mode for FdUrd and FU. The settings of the mass spectrometer for positive-ion mode were as follows: capillary voltage 4.0 kV; cone voltage 10.0 V; collision voltage 10 V. The settings of the mass spectrometer for negative-ion mode were as follows: capillary voltage 3.5 kV; cone voltage 30.0 V; collision voltage 15 V. For both positive-ion and negative-ion modes, the source temperature was set at 120 °C; the desolvation was set at temperature 450 °C, and the cone and desolvation gas flows were 50 and 550 L/h, respectively. Quadrupoles 1 and 3 each had low mass and high mass resolution set at 12.0. The dwell time was 0.2 s, and the interscan delay was 0.1 s. The span was set at 0 a.m.u. The MRM m/z transitions monitored were: positive 246>130 for FdCyd; 267>115 for [$^{15}\text{N}_2$, ^{13}C]-dFdC; negative 245>155 for FdUrd; 248>58 for [$^{15}\text{N}_2$, ^{13}C]-FdUrd; 129>41.2 for FU; and 131>42 for [$^{15}\text{N}_2$]-FU. The LC system and mass spectrometer were controlled by Waters MassLynx software (version 4.1), and data were collected with the same software.

2.4 Preparation of calibration standards and quality control samples

Stock solutions of analytes FdCyd, FdUrd, FU, and internal standards [$^{15}\text{N}_2$, ^{13}C]-dFdC, [$^{15}\text{N}_2$, ^{13}C]-FdUrd and [$^{15}\text{N}_2$]-FU, were prepared independently at 1 mg/mL in water and stored at -80°C . Working solutions of 0.1 mg/mL mix of FdCyd, FdUrd, and FU and 0.001 mg/mL mix of [$^{15}\text{N}_2$, ^{13}C]-dFdC, [$^{15}\text{N}_2$, ^{13}C]-FdUrd and [$^{15}\text{N}_2$]-FU were also prepared and stored at -80°C . On the day of assay, the 0.1 mg/mL mix of FdCyd, FdUrd, and FU was serially diluted (in steps of 10-fold) in water to obtain the lower calibration working solutions. These calibration working solutions were diluted in human plasma spiked with 10 μL of 10 mg/mL THU per mL of plasma to produce the following analyte concentrations: 5, 10, 30, 100, 300, 1000, 3000 ng/mL. For each calibration series, zero and blank samples were also prepared from 200 μL of control plasma.

Quality control (QC) stock solutions were prepared independently from separate weighings and stored at -80°C . These solutions were diluted in human plasma spiked with 10 μL of 10 mg/mL THU per mL of plasma to produce the following QC samples of either: QC Lower Limit (QCLL) 5 ng/mL, QC Low (QCL) 15 ng/mL; QC Mid (QCM) 150 ng/mL, and QC High (QCH) 2,500 ng/mL.

2.5 Sample preparation

Ten μL of 1 $\mu\text{g/mL}$ mix of [$^{15}\text{N}_2$, ^{13}C]-dFdC, [$^{15}\text{N}_2$, ^{13}C]-FdUrd and [$^{15}\text{N}_2$]-FU internal standards (IS) were added to 200 μL plasma sample. Proteins were precipitated with 1 mL of acetonitrile. Samples were vortexed for 1 min on a Vortex Genie-2 set at 10 (Model G-560 Scientific Industries, Bohemia, NY, USA) and then centrifuged at $12,000 \times g$ at room temperature for 5 min. The resulting supernatants were transferred to 12 mm $\times$ 75 mm borosilicate glass tubes and evaporated to dryness under a stream of nitrogen at 37°C . Dried residues were re-dissolved in 200 μL of acetonitrile and vortexed briefly. One hundred μL of the supernatants were transferred to auto-sampler vials and run on the FdUrd-FU assay that day or stored overnight at -20°C before running. For the FdUrd-FU assay, 20 μL of sample was injected into the LC-MS/MS system. The remaining 100 μL were dried down again, and then redissolved in 100 μL of water. Supernatants were transferred to auto-sampler vials and run on the FdCyd assay that day or stored overnight at -20°C before running. For the FdCyd assay, 10 μL of sample was injected into the LC-MS/MS system.

2.6 Validation procedures

2.6.1 Calibration curve and lower limit of quantitation (LLQ)—Decreasing concentrations of analytes were injected into the analytical system to determine the minimal concentration with a signal-to-noise ratio of at least 5:1. Calibration standards and blanks were prepared (see paragraph 2.4) and analyzed in triplicate to establish the calibration range with acceptable accuracy and precision. The analyte-to-internal standard ratio (response) was calculated for each sample by dividing the area of the analyte peak by the area of the internal standard peak. Standard curves were constructed individually by plotting the analyte-to-internal standard ratio *versus* the known concentrations in each sample. Standard curves were fitted by linear regression with weighting by $1/y^2$, followed by back-calculation of concentrations. The deviations of these back-calculated concentrations from the nominal concentrations were expressed as percentage of the nominal concentration.

2.6.2 Accuracy and precision—The accuracy and precision of the assay were determined by analyzing samples at the LLQ (QCLL), QCL, QCM, and QCH concentrations in 6 replicates each in 3 analytical runs, together with independently prepared, triplicate calibration curves. Accuracy was calculated at each test concentration as: (mean measured concentration/nominal concentration) $\times$ 100%.

Assay precision was calculated by ANOVA as previously described [12], by using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA). Back-calculated concentrations of calibration and QC samples were entered with the run number as factor. From the resulting mean squares of the within runs and mean squares of the between runs, the intra-assay and inter-assay precisions were calculated.

2.6.3 Selectivity and specificity—To investigate whether endogenous matrix constituents interfered with the assay, six individual batches of control, drug-free human plasma were processed and analyzed according to the described procedure. Responses of analytes at the LLQ concentrations were compared with the response of the blank samples. Relevant cross-talk of each individual analyte was characterized by detection on all other MRM channels by analysis of individual analytes and IS's at 3000 ng/mL.

2.6.4 Extraction recovery and ion-suppression—We determined the extraction recoveries of FdCyd and its metabolites from plasma by comparing the absolute response of an extract of control plasma to which these analytes had been added after extraction, with the absolute response of an extract of plasma to which the same amounts had been added before extraction. The ion-suppression by plasma matrix components was defined as the decrease of the absolute response of an extract of control plasma to which analyte had been added after the extraction relative to the absolute response of reconstitution solvent to which the same amount of each respective analytes had been added. Experiments were performed at the three QC concentrations, in quadruplicate.

2.6.5 Stability—Long-term stability experiments were performed in plasma and in stock solution after storage at -80°C . Stability in the stock solution was expressed as the percentage recovery of the stored solution (7 months) relative to a fresh solution. The stabilities of FdCyd and metabolites in plasma at -80°C were determined by assaying samples before and after storage. In addition, the stabilities of FdCyd and metabolites in stock solution at room temperature for 4 h were determined in triplicate. All stability testing in plasma was performed in quadruplicate at the QCL, QCM and QCH concentrations. For long-term stability, quadruplicate samples of the QCLL were also run. The effect of 3 freeze/thaw cycles analyte concentrations on plasma was evaluated by assaying samples after they had been frozen (-80°C) and thawed on 3 separate days and comparing the results with those of freshly prepared samples. The stabilities of FdCyd and metabolites in plasma during sample preparation were evaluated by assaying samples before and after 4 h of storage at room temperature. To evaluate the stabilities of FdCyd and metabolites in reconstituted samples in the autosampler, we re-injected QC samples and calibration curves approximately 72 h after the first injection and compared the concentrations derived from the second injection with those derived from the first injection.

2.6.6 Dilutional integrity—To demonstrate dilutional integrity, the ability to dilute samples from above the upper limit of quantitation to within the validated concentration range, plasma samples containing FdCyd and metabolites above the upper limit of quantitation were diluted to within the assay range. Plasma samples (N=4) with analyte concentrations of 20,000 ng/mL and 50,000 ng/mL were diluted 10-fold (to 2000 ng/mL) and 100-fold (to 500 ng/mL), respectively, with control plasma and assayed.

2.7 Application of the assay

To document the applicability of the assay, we present the pharmacokinetic profile of FdCyd and metabolites after administration of a 120 mg PO dose of FdCyd combined with a 3000 mg PO dose of THU to a patient enrolled on the ongoing phase I clinical trial of FdCyd ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01534598) Identifier: NCT01534598).

3 Results and Discussion

3.1 Validation of the assay

3.1.1 Chromatography—The approximate retention times of each compound were as follows: FdCyd at 2.7 min, FdUrd at 7.8 min, FU at 5.5 min, [$^{15}\text{N}_2$, ^{13}C]-dFdC at 2.6 min, [$^{15}\text{N}_2$, ^{13}C]-FdUrd at 7.8 min, and [$^{15}\text{N}_2$]-FU at 5.5 min.

The analytes at the retention time extremes of approximately 2.6 and 7.8 min, had corresponding capacity factors of 1.7 and 4.0, respectively, with a void time of 1.0 min. Representative chromatograms of each compound (at the LLQ), and internal standards in plasma are displayed in Fig. 2.

3.1.2 Calibration curve and LLQ—According to the FDA guidelines for bioanalytical method validation [13], the calibration curve adequately describes the concentration *versus* response relationship if the observed deviation and precision are $\leq 20\%$ for the LLQ and $\leq 15\%$ for all other calibration concentrations. At least 4 of 6 calibration points should meet the above criteria [13].

The selected assay range of 5–3000 ng/mL fulfilled the FDA criteria for the LLQ concentration and the calibration curve. Accuracies and precisions at the different concentrations were determined from triplicate calibration curves on 3 separate days and are reported in Table 1. At most concentrations, the mean square of the within runs was greater than the mean square of the between runs, indicating that there was no significant additional variability due to the performance of the assay in different runs [12]. Representative calibration curves and corresponding correlation and regression coefficients are shown in Fig. 3.

3.1.3 Accuracy and precision—FDA guidelines specify that the accuracies for all tested concentrations should be within $\pm 15\%$, and the precisions should not be $>15\%$ CV except for the LLQ, in which case these parameters should not exceed 20% [13].

The accuracies and intra- and inter-assay precisions for the tested concentrations (QCLL, QCL, QCM, QCH) were all within the defined acceptance criteria (Table 2).

3.1.4 Selectivity and specificity—According to FDA guidelines, the signal at the LLQ must be at least 5 times the signal of any co-eluting peaks [13].

Chromatograms of six individual control plasma samples contained no co-eluting peaks >20% of the analyte areas at the LLQ concentration (interference <2.1% for FdCyd, <12% for FdUrd, and <14% for FU (Fig. 2). We were not able to baseline separate FU from a closely eluting endogenous peak, however, manual integration by drop-down to baseline at the point of the inter-peak valley resulted in adequate performance.

Cross talk calculations were performed and revealed the following results: FU cross talks with FdUrd at 0.1% and FdUrd cross talks with FU at 0.01%. [$^{15}\text{N}_2$, ^{13}C]-dFdC IS cross talks with FdCyd at 0.02%, while FdCyd cross-talks with [$^{15}\text{N}_2$, ^{13}C]-dFdC IS at 0.02%. [$^{15}\text{N}_2$, ^{13}C]-FdUrd IS cross talks with FdUrd at 0.03%, while FdUrd cross-talks with [$^{15}\text{N}_2$, ^{13}C]-FdUrd IS at 0.04%. [$^{15}\text{N}_2$]-FU IS cross talks with FU at 0.05%, while FU cross-talks with [$^{15}\text{N}_2$]-FU IS at 0.07%. Cross talk does not appear to be a major issue with any of the analytes.

3.1.5 Extraction recovery and ion-suppression—As outlined in the FDA-guidelines, there is a requirement that recovery be consistent and precise [13]. A recovery of 70% with a variation of 15% is generally accepted [12, 13]. There is no specific requirement for ion-suppression. Ultimately, the assay performance, as expressed in the precision and accuracy, is most relevant; however, a large and/or variable ion-suppression may result in lack of assay robustness.

Recovery of FdCyd ranged from 105.6 to 156.1%, with CVs between 12.7% and 49.9%. The recoveries of FdUrd and FU ranged from 1.6 to 3.8%, with CVs between 8.8 and 60.1%. Ion-suppression ranged from 52.4 to 55.6% for FdCyd, with CVs between 33.2 and 67.3%, and 9.9 to -21.5% (i.e. ionization enhancement) for FdUrd and FU, with CVs between 1.5 and 9.6% (Table 3).

3.1.6 Stability—Stability in biological samples is acceptable when 85% of the analyte is recovered. The stabilities of FdCyd, FdUrd, and FU stock solutions at room temperature for 6 h were 100.3%, 101.0%, and 100.1%, respectively (Table 4). Stabilities in stock solutions for 7 months at -80 °C were 91.7%, 101.3%, and 100.4% for FdCyd, FdUrd, and FU, respectively. The stability of the analytes after 3 freeze thaw cycles (-80 °C to RT) were between 95.9 and 111.4%. Long-term stabilities of the analytes in plasma at -80 °C were between 91.6 and 105.7%. The absolute responses of plasma extracts of FdCyd at the QC concentrations, when reconstituted and kept in the autosampler for 72 h, were 96.2 to 110.5% of the initial responses (CV 5.00–9.71%). For the metabolites, the absolute responses of plasma extracts after 72 h, were 96.4 to 105.1% of the initial responses (CV 2.8–11.1%). Importantly, the reinjection run passed the requirements of any run set by the FDA [13].

3.1.7 Dilutional Integrity—The samples diluted from 50 µg/mL to 500 ng/mL displayed the following accuracies for each analyte: FdCyd: -4.1%, with a CV of 4.5%; FdUrd: -3.85%, with a CV of 5.66%; and FU: -0.075%, with a CV of 6.04%. The samples diluted

from 20 µg/mL to 2000 ng/mL displayed the following accuracies for each analyte: FdCyd: -9.39%, with a CV of 0.787%; FdUrd: -3.79%, with a CV of 3.74%; and FU: -5.95%, with a CV of 3.11%.

These results indicate dilutional integrity for all analytes.

3.2 Development

A previously described, but not fully validated, assay for the quantitation of FdCyd, FdUrd, FU, 5-fluorouridine (FUrd) and 5-fluorocytosine (FC) had been in use in our laboratory for a number of years [6, 8, 10, 11]. This assay utilized 100 µL of plasma for an ethyl acetate liquid-liquid extraction, followed by chromatography on 2 serial NH2P-50 2D HILIC columns, resulting in a run time of 49 minutes. Of the analytes monitored, FUrd was never observed as a metabolite of FdCyd, and FC has only been observed a day after PO FdCyd at low ng/mL concentrations. In this previous assay, FdCyd and FC were detected utilizing the same MRM channel of m/z 130 to 113, which for FdCyd represents in-source fragmentation to FC cross-talk. Typical dynamic ranges for each analyte were: FdCyd 10–5000 ng/mL, FdUrd 5–2500 ng/mL, FU 3–500 ng/mL, FUrd 30–5000 ng/mL, and FC 10–5000 ng/mL. Although fit-for-purpose for several years, over time, the lower limit of quantitation appeared to rise, and chromatography of especially the later eluting FdCyd and FC peaks was suboptimal due to broad peaks. Sudden loss of FdCyd sensitivity by a factor of 10-fold prompted us to re-develop the assay followed by extensive validation.

Because FUrd and FC were not considered clinically relevant metabolites of FdCyd, we removed these from our new assay. Next, we retuned on FdCyd utilizing MRM channel m/z 246 to 130, thereby precluding cross-talk from FC, even if formed after PO dosing of FdCyd. We decided to take away one of the NH2P-50 2D HILIC columns to shorten the original run time of 49 min. However, the peak area of FdCyd was inadequate at lower concentrations using the HILIC column. With a Phenomenex Synergi Polar RP column, the peak area was much higher and an adequate peak shape was obtained for FdCyd. The better response appeared to be due to the higher percentage of water in the mobile phase utilized for the Polar RP column, which was shown to improve FdCyd ionization. However, on the Polar RP column, plasma samples produced an interfering peak that exactly co-eluted with the FU peak, and the peak shape of FdUrd was asymmetric. The gradient mobile phase was varied in an attempt to better separate the endogenous plasma peak from FU and to improve the peak shape of FdUrd. These attempts proved to be futile and we therefore decided to employ 2 different chromatographic methods. The Phenomenex Synergi Polar RP column proved to be far superior to the HILIC column for FdCyd sensitivity and the HILIC column was better than the Phenomenex column for FU and FdUrd chromatography. Although baseline separation was not achieved for FU and the endogenous peak, separation was deemed sufficient to allow quantitation. As opposed to performing 2 completely separate assays to measure the compounds we decided to perform one protein precipitation per sample and then split the reconstituted supernatant to be analyzed with two 2 LC methods. Recovery of all analytes was variable (raw detector responses with approximately 35% CV, resulting in approximately 50% CV for the calculated recovery), and the recovery for FdUrd and FU was rather low at less than 5%. The latter result is likely due to the inability of 100%

acetonitrile to completely dissolve the dried residue of a 5:1 acetonitrile:plasma precipitation step. After aspirating 100 μ L of the 200 μ L of the acetonitrile primary reconstitution solvent for analysis of FU and FdUrd (recovery <5%), the remaining 100 μ L was dried down again, followed by reconstitution in 100% water for analysis of FdCyd (recovery ~100%). Based on the structural similarities, there is no reason to expect FdCyd to behave any different from FdUrd in terms of recovery, and the use of type of solvent for reconstitution would explain this discrepancy. We explored 5, 10, and 20% water in acetonitrile as primary reconstitution solvent, however, this resulted in progressively poor peak-shape at no improvement of sensitivity. Injecting less sample, 20, 5, and 1 μ L did not improve peak shape enough to make up for the lower sensitivity due to less amount injected on column. Having the benefit of stable-isotope internal standards and accurate and reproducible results (see Table 1 and Table 2), we did not further pursue the optimization of this assay component.

3.3 Application of the assay

As seen in Fig. 4, the assay was capable of quantitating FdCyd and metabolites in the patient samples available, allowing adequate evaluation of the pharmacokinetic and metabolic profile of FdCyd. Available clinical samples that had been analysed with the old assay, though beyond the documented stability period of 6 months, were analysed with the new assay. The relative differences observed were: FdCyd 30.4% (N=16, range 4.7% to 64.5%, N=6 <20% difference), FdUrd 10.5% (N=11, range -12.2% to 25.4%, N=9 <20% difference), and FU 32.1% (N=2, 28.9% and 35.3%).

3.4 Incurred sample reanalysis and external validation

Upon completion of the validation, incurred sample reanalysis for was performed on clinical samples from the ongoing clinical trial being supported with this assay ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01534598) Identifier: NCT01534598). Reanalysis yielded the following results: FdCyd, N=43, mean difference=0.86%, 100% of samples within 20%; FdUrd, N=38, mean difference=0.78%, 100% of samples within 20%; FU, N=34, mean difference=-1.0%, 82% of samples within 20%.

4 Conclusion

The objective of this study was to develop and validate an analytical method for the quantitation of FdCyd, FdUrd, and FU in human plasma to support ongoing clinical studies. We accomplished this using a single extraction step, followed by chromatography on two separate columns to allow for quantitative analysis of the three analytes of interest. A previously described, but not fully validated, assay for the quantitation of FdCyd, FdUrd, FU, 5-fluorouridine (FUrd) and 5-fluorocytosine (FC) had been in use in our laboratory for a number of years [6, 8, 10, 11]. Our new assay, though utilizing two chromatographic systems, is faster, covers a wider concentration range of the clinically observed analytes, and has been validated according to FDA guidance [7].

The analytical method presented has been a valuable tool in quantitating FdCyd and its metabolites in plasma, and will continue to support its further clinical development.

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HIGHLIGHT

- FdCyd is a DNA methylation inhibitor undergoing Phase I and II trials
- An LC-MS/MS assay from 5–3000 ng/mL in 0.2 mL plasma was validated
- Separate chromatography for FdCyd, and FdUrd and FU, yielded adequate performance
- This assay is being utilized to quantitate FdCyd, FdUrd, and FU in clinical samples

Fig. 1. Structures of FdCyd, its metabolites FdUrd (mediated by cytidine deaminase), and FU (mediated by thymidine phosphorylase) and their respective internal standards $[^{15}\text{N}_2, ^{13}\text{C}]$ -dFdC, $[^{15}\text{N}_2, ^{13}\text{C}]$ -FdUrd, $[^{15}\text{N}_2]$ -FU.

Fig. 2.

Representative chromatograms of: A) FdCyd (m/z 246>130; 2.7 min) added to control plasma at the LLQ concentration of 5 ng/mL (top trace with an offset of 100 counts) and control human plasma (bottom trace); B) FdUrd (m/z 245>155; 7.8 min) added to control plasma at the LLQ concentration of 5 ng/mL (top trace) and control human plasma (bottom trace); C) FU (m/z 129>41.2; 5.5 min) added to control plasma at the LLQ concentration of 30 ng/mL (top trace with an offset of 50 counts) and control human plasma (bottom trace); D) [$^{15}\text{N}_2$, ^{13}C]-dFdC internal standard (m/z 267>115; 2.6 min) added to control plasma at a concentration of 50 ng/mL (top trace with an offset of 100 counts) and control human

plasma (bottom trace); E) [$^{15}\text{N}_2$, ^{13}C]-FdUrd (m/z 248>158; 7.8 min) added to control plasma at a concentration of 50 ng/mL (top trace with an offset of 500 counts) and control human plasma (bottom trace); F) [$^{15}\text{N}_2$]-FU (131>42; 5.5 min) added to control plasma at a concentration of 50 ng/mL (top trace with an offset of 100 counts) and control human plasma (bottom trace).

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Fig. 3. Representative calibration curves ($N=3$ for each concentration) used to quantitate FdCyd ($\circ$), FdUrd ($\square$), and FU ($\triangle$) in human plasma samples (response FdCyd = $0.02847 \cdot \text{conc} + 0.00345$; $R^2=0.9909$, response FdUrd = $0.01156 \cdot \text{conc} - 0.01327$; $R^2=0.9966$, and response FU = $0.01181 \cdot \text{conc} - 0.00878$; $R^2=0.9922$). Calibration curves are depicted as response ratio *versus* nominal concentration (A), and as %residuals of the back-calculated relative to the nominal concentrations *versus* the log transformed concentration (B), the log-transformation for visual purposes.

Fig. 4.

Plasma concentrations of FdCyd (○), FdUrd (□), and FU (△) in a patient after oral administration of 120 mg FdCyd 30 min after 3000 mg of THU.

Table 1

Assay performance data of the calibration samples for FdCyd and its metabolites in human plasma.

Analyte	Conc. (ng/mL)	Accuracy (%)	Intra-assay precision (%)	Inter-assay precision (%)
FdCyd	5	104.8	13.6	*
	10	100.6	10.8	*
	30	103.0	8.6	*
	100	103.6	5.5	1.3
	300	103.8	6.3	*
	1000	99.9	5.6	*
	3000	95.8	4.1	1.0
FdUrd	5	102.2	4.7	*
	10	97.4	6.3	*
	30	99.7	5.9	*
	100	96.7	3.7	1.9
	300	103.1	2.4	*
	1000	103.6	2.9	*
	3000	103.4	3.5	*
FU	5	101.8	4.0	*
	10	98.3	8.9	*
	30	100.3	6.7	1.7
	100	99.6	4.0	*
	300	103.0	4.3	*
	1000	103.4	3.6	*
	3000	98.0	4.5	*

N=9; triplicate results, each in 3 separate runs, for each concentration.

* The mean square of the within runs was greater than the mean square of the between runs, indicating that there was no significant additional variation due to the performance of the assay in different runs [12].

Table 2

Assay performance data for the quantitation of QCLL, QCL, QCM and QCH FdCyd and its metabolites concentrations in human plasma.

Analyte	Concentration (ng/mL)	Accuracy (%)	Intra-assay precision (%)	Inter-assay precision (%)
FdCyd	5 (QCLL)	101.3	6.6	*
	15 (QCL)	104.3	4.4	2.3
	150 (QCM)	105.5	5.1	1.4
	2500 (QCH)	96.7	6.0	4.0
FdUrd	5 (QCLL)	100.7	6.8	2.5
	15 (QCL)	102.2	8.1	*
	150 (QCM)	102.7	5.0	*
	2500 (QCH)	102.2	3.0	*
FU	5 (QCLL)	101.3	7.4	*
	15 (QCL)	99.0	5.8	*
	150 (QCM)	105.1	4.2	1.8
	2500 (QCH)	99.8	3.7	2.4

N=18; 6-fold results, each in 3 separate runs, for each concentration.

* The mean square of the within runs was greater than the mean square of the between runs, indicating that there was no significant additional variation due to the performance of the assay in different runs [12].

Table 3

Recoveries of FdCyd and its metabolites from human plasma and their respective ion suppressions in human plasma extract, with coefficients of variation (CV).

Analyte	Concentration (ng/mL)	Recovery (%)	CV (%)	Ion suppression (%)	CV (%)
FdCyd	15 (QCL)	156.1	49.9	55.6	44.9
	150 (QCM)	105.6	12.7	54.1	33.2
	2500 (QCH)	126.9	49.0	52.4	67.3
FdUrd	15 (QCL)	1.60	28.2	9.9	1.5
	150 (QCM)	2.43	11.8	9.8	7.3
	2500 (QCH)	3.79	8.8	-1.1	1.5
FU	15 (QCL)	1.84	27.9	-10.0	4.2
	150 (QCM)	2.61	15.3	-9.2	9.6
	1500 (QCH)	3.81	60.1	-21.5	3.0

N=3, for each concentration.

Table 4

Stability of FdCyd and its metabolites under varying conditions.

Storage condition	Concentration (ng/mL)	Stability (%)	CV (%)	Replicates
FdCyd				
Stock solution 6 h				
Ambient temp.	100,000	100.3	4.2	3
Stock solution 7 months				
–80 °C	100,000	91.7	7.0	3
Plasma 4 h				
Ambient temp.	QCL 15	101.8	5.8	4
	QCM 150	91.2	3.4	4
	QCH 2500	100.8	4.3	4
Plasma 3 freeze-thaw cycles				
–80 °C	QCL 15	99.2	6.3	4
	QCM 150	98.5	6.6	4
	QCH 2500	97.8	5.2	4
Plasma 6 months				
–80 °C	QCLL 5	99.5	9.9	4
	QCL 15	89.0	5.0	4
	QCM 150	92.3	9.5	4
	QCH 2500	90.2	9.9	4
FdUrd				
Stock solution 6 h				
Ambient temp.	100,000	101.0	3.2	3
Stock solution 7 months				
–80 °C	100,000	101.3	3.1	3
Plasma 4 h				
Ambient temp.	QCL 15	101.6	5.7	4
	QCM 150	98.3	2.5	4
	QCH 2500	101.1	5.2	4

Storage condition	Concentration (ng/mL)	Stability (%)	CV (%)	Replicates
Plasma 3 freeze-thaw cycles				
-80 °C	QCL 15	100.4	7.9	4
	QCM 150	95.9	4.4	4
	QCH 2500	98.8	3.2	4
Plasma 6 months				
-80 °C	QCLL 5	95.2	2.8	4
	QCL 15	91.6	11.1	4
	QCM 150	102.3	6.1	4
	QCH 2500	105.7	3.3	4
FU				
Stock solution 6 h				
Ambient temp.	100,000	100.1	2.1	3
Stock solution 7 months				
-80 °C	100,000	100.4	4.3	3
Plasma 4 h				
Ambient temp.	QCL 15	98.0	9.6	4
	QCM 150	100.3	4.9	4
	QCH 2500	110.2	10.0	4
Plasma 3 freeze-thaw cycles				
-80 °C	QCL 15	103.3	9.9	4
	QCM 150	97.7	4.3	4
	QCH 2500	111.4	10.1	4
Plasma 6 months				
-80 °C	QCLL 5	96.3	10.6	4
	QCL 15	103.3	4.8	4
	QCM 150	104.5	6.9	4
	QCH 2500	103.8	3.9	4