Journal of Pharmaceutical and Biomedical Analysis 151 (2018) 25–31

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

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

M. van Nuland[a,d,*], M.J.X. Hillebrand[a], H. Rosing[a], J.A. Burgers[b], J.H.M. Schellens[c,d,e], J.H. Beijnen[a,d,e]

[a] Department of Pharmacy & Pharmacology, Antoni van Leeuwenhoek/The Netherlands Cancer Institute and MC Slotervaart, Amsterdam, The Netherlands
[b] Department of Thoracic Oncology, The Netherlands Cancer Institute, Amsterdam, The Netherlands
[c] Division of Clinical Pharmacology, Department of Medical Oncology, Antoni van Leeuwenhoek/The Netherlands Cancer Institute, Amsterdam, The Netherlands
[d] Division of Pharmacoepidemiology and Clinical Pharmacology, Faculty of Science, Department of Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands
[e] Division of Pharmacology, Antoni van Leeuwenhoek/The Netherlands Cancer Institute, Amsterdam, The Netherlands

ARTICLE INFO

Article history:
Received 16 October 2017
Received in revised form
15 December 2017
Accepted 22 December 2017
Available online 26 December 2017

Keywords:
Gemcitabine
dFdU
microdose
Pharmacokinetics
Validation
LC–MS/MS

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

* Corresponding author at: Department of Pharmacy & Pharmacology, Antoni van Leeuwenhoek, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, the Netherlands.
E-mail address: m.v.nuland@nki.nl (M. van Nuland).

https://doi.org/10.1016/j.jpba.2017.12.048
0731-7085/

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)