Journal of Pharmaceutical and Biomedical Analysis 142 (2017) 125–135

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Development, validation and application of a novel liquid chromatography tandem mass spectrometry assay measuring uracil, 5,6-dihydrouracil, 5-fluorouracil, 5,6-dihydro-5-fluorouracil, α-fluoro-β-ureidopropionic acid and α-fluoro-β-alanine in human plasma

Ottiniel Chavani[a,*], Berit Packert Jensen[a], R. Matthew Strother[b], Christopher M. Florkowski[a], Peter M. George[a]

[a] Canterbury Health Laboratories, Christchurch, New Zealand
[b] Oncology, Christchurch Hospital, Christchurch, New Zealand

ARTICLE INFO

Article history:
Received 24 November 2016
Received in revised form 23 April 2017
Accepted 24 April 2017
Available online 6 May 2017

ABSTRACT

The plasma 5,6-dihydrouracil/uracil (UH_2/U) ratio is a possible phenotypic marker of dihydropyrimidine dehydrogenase (DPD) activity, hence an index of 5-fluorouracil (5-FU) response and toxicity. Studies have re-affirmed the value of 5-FU and 5,6-dihydro-5-fluorouracil (FUH_2) for therapeutic drug monitoring (TDM). However, FUH_2 has limited stability in plasma, necessitating expedited plasma separation and freezing, where routine compliance may not be easy. The metabolites α-fluoro-β-ureidopropionic acid (FUPA) and α-fluoro-β-alanine (FβAL) are stable in plasma and are probable candidates for TDM. This paper describes development, validation and application of an LC–MS/MS assay quantifying U, UH_2, 5-FU, FUH_2, FUPA and FβAL in human plasma.

Extraction was by salt-assisted liquid–liquid extraction (LLE) in two-stages with pH adjustment. The supernatants were mixed, dried and reconstituted. Analytes were resolved on the Luna PFP (2) (150 × 2.00 mm, 3 μ) column by gradient elution and analyzed by tandem mass spectrometry via electrospray ionisation in positive polarity.

1. Introduction

5-FU, a pyrimidine analogue, is a commonly used chemotherapeutic in solid tumours of the digestive tract, breast plus head and neck [1]. Its administration is typically intravenous bolus or continuous infusion. Most current dosing algorithms use body surface area to normalize exposure. However, >50% of patients have plasma 5-FU values outside the optimal range [2] and up to 100-fold individual variability in pharmacokinetic parameters have been observed [3–5]. An association between 5-FU pharmacokinetic parameters and efficacy plus toxicity has been reported and optimal therapeutic windows have been proposed [6–8].

Variability in 5-FU exposure has been associated with differences in the three-step sequential metabolic pathway which rapidly degrades approximately 80% of systemic 5-FU [9] (Fig. 1) and is partially attributable to polymorphisms, environmental exposures or comorbidity. Diminished metabolism augments systemic 5-FU exposure which compromises efficacy and occasionally leads to toxicity. DPD is the initial, rate-limiting and saturable enzyme but variability in DHPase and β-alanine synthase may also modify toxicity risk [10–14].

To minimize toxicity, a two-step strategy is needed: prediction of first dose, followed by TDM for subsequent doses. Response and toxicity are known to be improved by pharmacokinetic adjustment of 5-FU dose [7,15,16]. However, generally the first dose is determined using body surface area, thus possibly not achieving optimal therapeutic concentration and is potentially toxic. At present, there is no universally applied *a priori* test for identification of patients with an increased risk of toxicity or treatment efficacy.

The endogenous plasma UH_2/U ratio is a reasonable index of response and toxicity. A high degree of concordance between elevated 5-FU and low endogenous UH_2/U is known [17]. Hence it

* Corresponding author at: Toxicology, Canterbury Health Laboratories, P.O. Box 151, Christchurch 8140, New Zealand.
E-mail address: Ottiniel.Chavani@cdhb.health.nz (O. Chavani).

http://dx.doi.org/10.1016/j.jpba.2017.04.055
0731-7085/

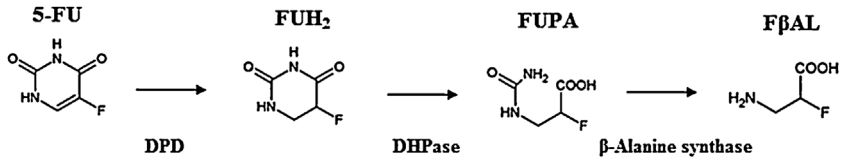

Fig. 1. 5-FU metabolic pathway.

is postulated that a first dose based on UH_2/U will improve the proportion of individuals achieving therapeutic concentration and diminish early toxicity events. However, the finding of elevated 5-FU in patients with normal UH_2/U ratio [17] illustrates the limitation of this approach. In order to further investigate the UH_2/U ratio plus TDM of 5-FU and its metabolites to improve the dosing of 5-FU, an assay for measuring U, UH_2, 5-FU, FUH_2, FUPA and FβAL in plasma is required for clinical studies as well as future clinical application.

In the literature, numerous assays have been described measuring 5-FU and FUH_2 for TDM using immunoassays [18], HPLC [19,20] and LC–MS/MS [9,21–23]. However, the superiority of LC–MS/MS in context of selectivity and sensitivity is well-known. Assays for the measurement of the metabolites FUPA and FβAL involve analyte derivatization [24–27], a combination of LLE with solid phase extraction [27] and protracted protein precipitation with sulfosalicylic acid [26]. For U and UH_2, there have been several published LC–MS/MS methods [17,22,28,29]. To our knowledge, no assay, however has been reported for a combination of the 6 analytes (U, UH_2, 5-FU, FUH_2, FUPA and FβAL). A multi-analyte assay would provide operational advantages including simplified workflow, reduced labour and analysis time, as well as reduced requirements for volumes of sample and reagents. In order to further rationalize 5-FU dosing, we report development, validation and clinical application of an LC–MS/MS assay quantifying U, UH_2, 5-FU, FUH_2, FUPA and FβAL in human plasma.

2. Materials and methods

2.1. Chemicals and materials

U ($C_4H_4N_2O_2$), U-IS ($C_3{}^{13}CH_4{}^{15}N_2O_2$), UH_2 ($C_4H_6N_2O_2$), UH_2-IS ($C_3{}^{13}CH_6{}^{15}N_2O_2$), 5-FU ($C_4H_3FN_2O_2$), 5-FU-IS ($C_3{}^{13}CH_3F^{15}N_2O_2$), FUH_2 ($C_4H_5FN_2O_2$), FUH_2-IS ($C_3{}^{13}CH_5F^{15}N_2O_2$), FUPA ($C_4H_7FN_2O_3$), FUPA-IS ($C^{13}C_3H_7FN_2O_3$) and FβAL ($C_3H_6FNO_2$) with purity ≥96% were purchased from Toronto Research Chemicals (North York, Canada) and FβAL-IS ($C_3H_3D_3F^{15}NO_2$) with purity of 98% was supplied by Alsachim (Strasbourg, France). Acetonitrile, methanol, ethyl acetate and 2-propanol (HPLC grade or better), acetic acid (Suprapur grade) and $ZnSO_4$ were obtained from Merck (Darmstadt, Germany). Bovine serum albumin (BSA) was supplied by Life Technologies Corporation (Auckland, New Zealand). Delipidized human plasma (VD-DCC Mass Spect Gold®) was supplied by Golden West Biologicals, Inc.® (Temecula, USA).

2.2. Preparation of calibration curves, quality control samples and internal standard

Two individual stock solutions of each of 5-FU, FUH_2, FUPA and FβAL at 1 mg/mL; U and UH_2 at 0.1 mg/mL from independent weighings were prepared in 50% methanol and stored at −80 °C. All stable isotope-labeled IS stock solutions were made at 1 mg/mL in 50% methanol and stored under the same conditions. The working IS concentrations (ng/mL) were 250, 5-FU and FUPA; 500, FUH_2 and FβAL; 50, U; and 100, UH_2 in water.

Calibration curves containing (ng/mL) 50, 5000, 10 000, 20 000, 50 000, and 100 000 for 5-FU; 50, 250, 500, 1000, 2500 and 5000 for FUPA; 50, 500, 1000, 2000, 5000 and 10 000 for FUH_2 and FβAL; 5, 10, 20, 40, 100 and 200 for U and 10, 20, 40, 80, 200 and 400 for UH_2 were prepared freshly in 0.4% BSA. Delipidized plasma was spiked to obtain the concentrations (ng/mL) 150, 18750, 37500 and 75 000 for 5-FU, 150, 1875, 3750 and 7500 for FUH_2 and FβAL; 150, 1125, 2250 and 4500 for FUPA; 15, 37.5, 75 and 150 for U and 30, 75, 150 and 300 for UH_2 corresponding to lower quality control (LQC), inferior medium quality control (MQC1), superior medium quality control (MQC2) and high quality control (HQC).

2.3. Sample preparation

A 100 μL aliquot of working IS was added to 50 μL of sample in 2 mL polypropylene micro centrifuge tubes. Plasma proteins were precipitated with 100 μL of 1 M $ZnSO_4$ followed by 600 μL acetonitrile/methanol (95/5, v/v). Then, 50 μL of 0.25 M acetic acid and 400 uL of ethyl acetate/IPA (85/15, v/v) were added, mixed and centrifuged for 5 min at 16,000*g*. The supernatant was recovered into glass tubes.

To the sediment, 100 μL of $ZnSO_4$, 600 μL of acetonitrile/methanol, 50 μL of 1.0 M acetic acid and 400 μL of ethyl acetate/IPA were added concurrently, mixed and centrifuged as prior. The supernatants were mixed, dried in a CentriVap® Centrifugal Vacuum Concentrator (Labconco Corporation, Kansas, USA) at 50 °C and reconstituted in 50 μL of eluent A (defined in 2.4), vortexed and transferred to polypropylene vials.

2.4. Liquid chromatography

LC was performed on the 1290 Infinity system (Agilent Technologies, Santa Clara, USA (Agilent)) composed of a 1290 Infinity binary pump, degasser and thermostatted column compartment. Analytes were resolved on the Luna PFP (2) (150 × 2 mm, 3 μm) column protected by a PFP (2) guard column (4 × 2.0 mm, 3 μm) (Phenomenex, Torrance, USA).

Eluent A was acetic acid/formic acid/water (0.25/0.05/99.7, v/v/v) and B was acetonitrile. The following gradient was utilised: 0% B from 0 to 3 min, 0–90% B from 3.0–3.2 min, 90% B from 3.5–4.5 mins, 90–0% B from 4.5–5 mins and 0% B from 5 to 8 mins. The flow rate was 0.3 mL/min and eluent was directed to MS between 1.15 and 4.0 mins or otherwise to waste. The column was maintained at 30 ± 0.2 °C and injection volume was 10 μL.

2.5. Mass spectrometry

The MS analyses were performed on the 6460 Triple Quadrupole MS/MS system (Agilent) using positive electrospray ionization mode. A capillary voltage of 1750 V, gas temperature of 350 °C, gas flow of 10 L/min, sheath gas temperature of 400 °C, sheath gas flow of 10 L/min, nebulizer pressure of 45 psi and electron multiplier voltage of 400 V were used. Adjunct optimised parameters are shown in Table 1 along with suggested fragmentation as previously reported [22,30–32]. Data acquisition was via dynamic multiple reaction monitoring with resultant dwell times of 30–91 microseconds across the analyte range. MassHunter Workstation Software, *Version* B.06.00 (Agilent) was used for operating hardware, data acquisition and integration.