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Simultaneous quantification of busulfan, clofarabine and F-ARA-A using isotope labelled standards and standard addition in plasma by LC–MS/MS for exposure monitoring in hematopoietic cell transplantation conditioning

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ABSTRACT

In allogeneic hematopoietic cell transplantation (HCT) it has been shown that over- or underexposure to conditioning agents have an impact on patient outcomes. Conditioning regimens combining busulfan (Bu) and fludarabine (Flu) with or without clofarabine (Clo) are gaining interest worldwide in HCT. To evaluate and possibly adjust full conditioning exposure a simultaneous analysis of Bu, F-ARA-A (active metabolite of Flu) and Clo in one analytical run would be of great interest. However, this is a chromatographical challenge due to the large structural differences of Bu compared to F-ARA-A and Clo. Furthermore, for the bioanalysis of drugs it is common to use stable isotope labelled standards (SILS). However, when SILS are unavailable (in case of Clo and F-ARA-A) or very expensive, standard addition may serve as an alternative to correct for recovery and matrix effects. This study describes a fast analytical method for the simultaneous analysing of Bu, Clo and F-ARA-A with liquid chromatography-tandem mass spectrometry (LC–MS/MS) including standard addition methodology using 604 spiked samples. First, the analytical method was validated in accordance with European Medicines Agency guidelines. The lower limits of quantification (LLOQ) were for Bu 10 µg/L and for Clo and F-ARA-A 1 µg/L, respectively. Variation coefficients of LLOQ were within 20% and for low medium and high controls were all within 15%. Comparison of Bu, Clo and F-ARA-A standard addition results correspond with those obtained with calibration standards in calf serum. In addition for Bu, results obtained by this study were compared with historical data analysed within TDM. In conclusion, an efficient method for the simultaneous quantification of Bu, Clo and F-ARA-A in plasma was developed. In addition, a robust and cost-effective method to correct for matrix interference by standard addition was established.

1. Introduction

Allogeneic hematopoietic cell transplantation (HCT) is a last resort for a variety of malignant and nonmalignant disorders (e.g. immunodeficiencies and inherited metabolic diseases). Prior to HCT, preparative conditioning is incorporated to enable engraftment of donor cells. Although no consensus on optimal conditioning exists, the combination of Bu, F-ara-A-monophosphate (fludarabine, Flu) with or without Clo is used more and more in current HCT conditioning [1]. For Bu a target exposure has been defined [2,3]. However, for Clo and Flu including its active metabolite F-ara-A limited pharmacokinetic/pharmacodynamics (PKPD) data in the HCT setting are available. Recent reports do suggest that overexposure to Flu or Clo may be a predictor of poor transplant outcomes. Yet, optimal exposure to Flu and Clo is dependent on underlying disease and the selected conditioning regimen among others making it difficult to define a therapeutic window [4–6]. As therapeutic drug monitoring (TDM) of Bu is rapidly gaining interest worldwide, numerous bioanalytical methods predominantly using a stable isotope labelled (SIL) internal standard (IS) are available in literature [7–10]. For quantification of Clo combined with F-ARA-A in plasma an analytical method was published recently [11]. For the latter method 2-chloroadenosine was used as an IS, since SIL variants of Clo and SIL-F-ARA-A are not commercial available. To correct for matrix interference for Clo and F-ARA-A, the use of standard addition commonly used in pesticide analysis may serve as an alternative, [12]. Standard addition methodology can be employed to correct for the whole process efficacy including matrix effects and recovery losses, without the need for expensive labelled standards. In contract to pesticide analysis, the expected concentration range of the analytes is known and relatively small facilitating addition of optimal selected spike concentrations.

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Following their combined use and concomitant administration in the clinic, a simultaneous analysis of Bu, F-ARA-A and Clo in one analytical run would be of great interest to determine the optimal exposure for HCT. The latter can be considered a chromatographical challenge due to the large structural differences of Flu and Clo compared to Bu. The lipophilicity of Bu (0.5 logP) is more suitable for reverse phase chromatography whereas the 1.5 logP of F-ARA-A fits a normal phase chromatography (HILIC). Therefore, we aimed to develop a combined and fast analytical method for simultaneous quantification of Bu, clo, F-ARA-A in plasma with LC–MS/MS.

2. Material and method

2.1. Chemicals and reagents

All chemicals and compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless stated differently. Gibco Newborn Calfs Serum, heat inactivated, was obtained from Thermo Fisher Scientific (Waltman, MA, USA). Drug Free Serum was obtained from Bio-Rad (Hercules, CA, USA). Blank bovine serum was obtained from Drug analysis and toxicology (KKGT) studies (SKML, Nijmegen, Netherlands). Random blank patient plasma samples were randomly selected from residual material obtained in routine TDM specimens with patients consent.

2.2. Patients samples

After collection of blood, samples were stored in the refrigerator, plasma samples were obtained after centrifugation at the same day or the next day if samples were taken after working hours. After centrifugation samples were directly analysed and stored in the −80 °C freezer. Samples stored in the freezer (−80 °C) from pharmacokinetic studies in patients undergoing HCT conditioning with Bu, F-ARA-A combined with or without Clo and/or ATG were used for this study [13,14]. From January 2012 till December 2015 four time points were routinely drawn for the purpose of Bu TDM (t = 5 min, t = 1 h, t = 2 h and t = 3 h) following infusion of Bu. Between December 2015 and September 2016 Clo and F-ARA-A were included in the pharmacokinetic study and therefore an additional sample was taken between the end of Flu infusion and the start of Bu infusion (t = −3 h) in each patient. All included patients received Bu and Flu, and in 20% of the patients Clo was administered prior to infusion of both Flu and Bu. The study was approved by the local medical ethical committee. Broad informed consent was obtained from all patients.

2.3. Sample preparation

Plasma samples (50 µL) were pipetted into 1.5 mL eppendorf tubes and in order of succession the following solvents were added; 12.5 µL IS dissolved in acetonitrile (ACN): H_2O (1:1), 12.5 µL solvent ACN: H_2O (1:1) and 25 µL TCA (20%) and vortexed for 60 s. Thereafter, specimens were centrifuged for 5 min at 10.000*g*. Finally, 60 µL of the supernatant was transferred into glass vials with 540 µL 5% ACN.

2.4. Validation

Validation was performed in accordance with European Medicines Agency (EMA) guidelines [15]. For Bu, Clo and F-ARA-A separate stock solutions were made for calibration curves (CC) and quality controls (QC). CC and QC's were freshly prepared on each instance. For the CC eight levels of Bu were made by dilution in N,N,-Dimethylacetamide and for Clo and F-ARA-A in ACN/H_2O (1:1). For Bu, Clo and F-ARA-A 10 µL of each level of the standard solution was added to 970 µLblank calf serum. Standard calibration concentrations for Bu were 10, 50, 250, 1000, 5000, 7500 and 10000 µg/L and for Clo and F-ARA-A 1, 5, 25, 100, 500, 1000, 2500 and 5000 µg/L. Bu-D8 (10000 µg/L) and 2-chloroadenosine (2000 µg/L) dissolved in ACN/H_2O (1:1) were used as IS for respectively Bu of F-ARA-A and Clo. For accuracy and precision testing QC samples for Bu were made at lower limit of quantification (LLOQ) (10 µg/L), low (25 µg/L), medium (4000 µg/L) and high (8000 µg/L) in calf serum and for Clo and F-ARA-A respectively 1 µg/L, 2.5 µg/L, 2000 µg/L and 4000 µg/L were used as QC's. For selectivity testing, three samples obtained from an interproficiency testing program and 4 samples obtained from other pharmacokinetic studies were analysed (Section 2.1).

For stability testing at room temperature, 18 patient samples were selected, 12 samples contained Bu and F-ARA-A and 6 samples contained all three compounds. These samples were stored in a fume hood and four time points were taken at 0 h, 24 h, 48 h and 72 h. Autosampler stability was tested by analysing standard level 3 in calf serum every hour for a total 68 h. Long-term stability and stability after an extra freeze thaw cycle was tested when samples were stored at −80 °C. For F-ARA-A long-term stability was tested for 6 months and for Clo for 4 months. In addition, Bu concentrations were compared with historical routine monitoring results analysed with an in house prospectively validated LC–MS/MS method.

Statistical analyses for within run coefficient of variation (CV), between run CV and overall CV were performed using one-way analysis of variance (ANOVA). Linear regression analysis was used for autosampler stability. For comparing results obtained by standard addition with calibration curve, Deming regression was used in EP Evaluator (Build 10.3.0.556).

2.5. Standard addition

Patient samples were spiked to study recovery, ion suppression and matrix effects, Bu (5000 µg/L), Clo (200 µg/L) and F-ARA-A (1000 µg/L) were added to 604 samples. The primary goal of standard addition was optimal correction for potential matrix effects of Clo and F-ARA-A. For Bu, a stable labelled D8 Bu was used as an IS. Therefore, T = −3 samples (taken between December 2015 and September 2016) or T = 5 samples (taken from January 2012 till December 2015) were spiked. These samples were prepared in duplicate, by spiking one sample by replacing 12,5 µL ACN:H_2O (1:1) (Section 2.3) with 12,5 µL spike solution made in ACN:H_2O (1:1).

2.6. Instrumentation

All samples were analysed with an Ultimate 3000 UHPLC Dionex (Sunnyvale, CA) coupled to a triple quadrupole TSQ Quantiva, Thermo Fisher Scientific (Waltman, MA, USA). The method was validated with the following settings: 3 µL sample was injected onto an UPLC Acquity (BEH 2.1 * 50 mm, 1.7 µm particle size) analytical column (column temperature 40 °C), Waters (Milford, MA, USA). Eluents were 0.1% formic acid in water (eluent A) and ACN with 0.1% ammonium acetate (eluent B). The eluent profile consisted of 0–2.4 min isocratic 5% B, 2.4 − 2.75 min linear gradient from 5 to 95% B, 2.75–3.5 min isocratic gradient 95% B, 3.5 − 4.0 min linear gradient from 95 to 5%, and 4.0–4.5 min isocratic gradient 5% B, used flow rate was 0.7 mL/min. During method development different columns were tested (Atlantis Hilic 3 µm 2.1 × 100 mm (Waters), Hypersil GOLD PFP 5 µm 2.1 × 50 mm (Thermo Scientific), Kinetex Biphenyl 2.6 µm 2.1 × 50 mm (Phenomenex)). Based on retention times and peak shapes the above settings were used. Compounds were analysed by selected reaction monitoring (SRM) with the follow transitions: Bu 264 > 151.2 *m/z* (12 CE, 60 RF), Bu-D8 272 > 159 *m/z* (12 CE, 60 RF) F-ARA-A 286.1 > 154.1 *m/z* (17 CE, 49 RF) and 286.1 > 134.1 *m/z* (38 CE, 49 RF), Clo 304.1 > 107.1 *m/z* (23 CE, 77 RF) and 304.1 > 134.1 *m/z* (39 CE, 77 RF) and 2-cloroadenosine 302.1 > 170 *m/z* (21 CE, 62 RF) and 302.1 > 134.1 *m/z* (30 CE, 62 RF). Analytes were quantified with the follow MS conditions: in positive mode (3700 V), ion transfer tube temperature of 250 °C and vaporizer