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# Simultaneous determination of fludarabine and clofarabine in human plasma by LC–MS/MS

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ABSTRACT

A method for quantification of fludarabine (FDB) and clofarabine (CFB) in human plasma was developed with an API5000 LC–MS/MS system. FDB and CFB were extracted from EDTA plasma samples by protein precipitation with trichloroacetic acid. Briefly, 50 μL plasma sample was mixed with 25 μL internal standard (50 ng/mL aqueous 2-Cl-adensosine) and 25 μL 20% trichloroacetic acid, centrifuged at 25,000 × *g* (20,000 rpm) for 3 min, and then transfered to an autosampler vial. The extracted sample was injected onto an Eclipse extend $C_{18}$ column (2.1 mm × 150 mm, 5 μm) and eluted with 1 mM $NH_4OH$ (pH 9.6) – acetonitrile in a gradient mode. Electrospray ionization in positive mode ($ESI^+$) and multiple reaction monitoring (MRM) were used, and ion pairs 286/134 for FDB, 304/170 for CFB and 302/134 for the internal standard were selected for quantification. The retention times were typically 3.72 min for FDB, 4.34 min for the internal standard, 4.79 min for CFB. Total run time was 10 min per sample. Calibration range was 0.5–80 ng/mL for CFB and 2–800 ng/mL for FDB. The method was applied to a clinical pharmacokinetic study in pediatric patients.



## 1. Introduction

Allogeneic hematopoietic cell transplantation (alloHCT) has become the standard-of-care treatment for a variety of pediatric diseases, including leukemias, immunodeficiencies, and hemoglobinopathies. Although major advancements have been made in recent years through improvements in supportive care, for children with non-malignant disorders and certain myeloid malignancies high rates of engraftment failure and disease relapse remain prominent clinical problems. One of the most common conditioning regimens used in these children prior to alloHCT consists of fludarabine (FDB) combined with the alkylating agent, busulfan [1,2]. The addition of low-dose clofarabine (CFB) added to standard FDB and busulfan is being evaluated for safety and efficacy in a phase II trial (NCT01596699). CFB, like FDB, is a nucleoside analog with potent antitumor and immunosuppressive properties [3–5]. At low concentrations, the combination of CFB, FDB, and busulfan showed a higher degree synergistic cytotoxicity when compared with either nucleoside alone in combination with busulfan [6]. Given that both drugs share a similar metabolic pathway, drug-drug interactions may impact pharmacokinetics (PK) and drug disposition through several mechanisms, including altered drug clearance via renal elimination. Currently, no PK data are available for a combination nucleoside analog regimen containing both CFB and FDB to help inform optimal combination therapy. Such studies are limited by blood volume restrictions in children and dependent on a more sensitive, specific assay of CFB in plasma than those previously published [7].

A number of methods have been reported for the determination of FDB [8–10] and CFB [11–13]. However, methods for the simultaneous determination of these two nucleoside analogs have not yet been reported. Here we report an LC–MS/MS method for the simultaneous determination of FDB and CFB in human plasma with only a 50 μL sample, using 2-chloroadenosine as the internal standard (IS). Based on the regimen of the intended clinical study (40 mg/m$^2$ infusion of FDB over 1hr followed by 10 mg/m$^2$ infusion of CFB over 2 h) and published PK studies [7,14], we expect the minimal concentration in plasma for FDB and CFB will be >1 ng/mL and <1 ng/mL, respectively. Additionally, the anticipated maximum concentration will be around 1000 ng/mL for FDB and 100 ng/mL

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for CFB. Therefore we aim to develop a method with the calibration range at 2–800 ng/mL for FDB and 0.2–80 ng/mL for CFB.

## 2. Experimental

### 2.1. Chemicals and reagents

FDB and CFB (Fig. 1) were purchased from A.K Scientific Inc. (Mountain View, CA, USA); 2-chloroadenosine was from Sigma–Aldrich (St Louis, MO, USA). Acetonitrile (Optima™ LC/MS), water (Optima™ LC/MS), 21% ammonium hydroxide (Optima™), and trichloroacetic acid (Certified ACS) were obtained from Thermo-Fisher Sci. (Fair Lawn, NJ, USA). Mobile phase A was prepared by dissolving 91 μL 21% ammonium hydroxide ($NH_4OH$) in 1 L water; 20% trichloroacetic acid (TCA) was prepared by dissolving 2 g TCA in 10 mL water.

### 2.2. Instrumental

An AB Sciex API5000 was coupled with Shimadzu Prominence 20AD$^{XR}$ UFLC pumps and SIL-20AC$^{XR}$ autosampler and managed with the software Analyst® 1.5.1. The gases for the MS system were supplied by an LC–MS gas generator (Source 5000™, Parker Balston Inc., Haverhill, MA, USA). LC conditions were as follows: Separation was achieved on a Zorbax Extend $C_{18}$ (2.1 mm × 150 mm, 5 μm, Agilent Tech. Inc., Santa Clara, CA, USA) equipped with a guard column (12.5 mm × 2.1 mm, 5 μm) from the same source. Mobile phase A was 1 mM $NH_4OH$ and B was acetonitrile (MeCN). One microliter sample was injected onto the column eluted at a flow rate of 0.4 mL/min in a gradient program consisting of 4% solvent B (0–1 min), from 4 to 30% B (2–5 min), from 30 to 90% B (5–5.1 min), 90% B (5.1–6 min), 90–4% B (6.0–6.1 min), and 4% B (6.1–10 min). Retention times for CFB, FDB and the IS were 4.7 min, 3.6 and 4.2 min, respectively. Needle wash solvent was 50% MeCN. The divert valve was set to direct LC eluent to mass spectrometer (MS) source at 2 min and to waste line at 5.9 min. The MS conditions for FDB, CFB, and the IS were optimized by separate infusion of 50 ng/mL corresponding drugs into the MS at a flow rate of 10 μL/min constantly while adjusting MS parameters with autotune followed by manual adjustment to achieve maximal signal. The ion pairs 286/134 for FDB, 304/170 for CFB, and 302/134 for the IS were used for quantification in multiple reaction monitoring (MRM) mode. The optimized compound-dependent MS parameters were 56 v (DP), 55 v (CE), 12 v (CXP) for FDB ion pair 286/134, 101 v (DP), 28 v (CE), 24 v (CXP) for CFB ion pair 304/170, and 86 v (DP), 55 v (CE), 10 v (CXP) for the IS ion pair 302/134, respectively. DP was declustering potential, CE was collision energy, and CXP was collision cell exit potential; entrance potential was 10 v for all ion pairs. The MS parameters were optimized to maximize signal for CFB because of higher sensitivity requirement. The optimized MS parameters were as follows: MS source was TurboIon Spray ionization in positive mode ($ESI^+$) with turbo heater set at 600 °C; curtain gas was nitrogen at 40 psi, nebulizer gas (gas1) and auxiliary (Turbo) gas (gas 2) were zero air both at 60 psi, and collision-deactivated association gas was nitrogen at 9 psi; ionspray voltage was 2000 v. Data was processed with Analyst 1.5.1 (AB Sciex, Foster City, CA, USA).

### 2.3. Preparation of calibrators and QC samples

FDB and CFB primary stock solutions were prepared in 25% MeCN separately and diluted to prepare combined working solutions, which were spiked into EDTA human plasma to make calibrators of FDB/CFB at 2/0.2, 5/0.5, 10/1, 50/5, 100/10, 400/40, and 800/80 ng/mL and QC samples at 6/0.6, 60/6, and 700/70 ng/mL. Stock solution of 2-chloroadenosine (the IS) was prepared in 50% MeCN, which was serially diluted in water to make 50 ng/mL IS working solution. The prepared solutions and plasma samples were stored at −70 °C until use.

### 2.4. Sample preparation

Plasma samples (50 μL) were pipetted into 1.5 mL polypropylene eppendorf tubes, to which were added 25 μL IS (50 ng/mL aqueous 2-chloro-adenosine) and 25 μL 20% TCA. After vortexing, the samples were centrifuged at 25,000 × *g* for 3 min and 60 μL of the supernatant was transferred to an autosampler vial. The injection volume was 1 μL.

### 2.5. Validation

The method was validated according to the guidelines of NIH-sponsored AIDS Clinical Trial Group Network [15], which was based on FDA guidelines [16]. One set of calibrators were processed for each run and injected at the beginning and end. Calibration curves were constructed by linear regression of the peak area ratio of analyte to internal standard (*Y*-axis) versus the nominal analyte concentrations (*X*-axis) with a weighting factor of $1/x$. Precision was reported as relative standard deviation (RSD) and accuracy as percent deviation of the nominal concentration (% dev). The lower limit of quantification (LLOQ) was established with precision <20% and accuracy ±20%. Intra-day precision and accuracy were determined by analysis of at least five replicates of each QC sample at low (0.6/6 ng/mL), medium (6/60 ng/mL), and high (70/700 ng/mL) concentration levels extracted with a set of calibrators in one batch. The same procedure was repeated on at least 2 different days with new samples to determine inter-day precision and accuracy (total: $n \geq 15$ per concentration level).

Recovery and matrix effect was evaluated according to the approach published by Matuszewski et al. [17] Three sets of validation samples at low, medium, and high concentration were prepared. Set 1 samples were prepared by spiking both drugs (FDB/CFB) in 5%TCA in water at 3/0.3, 30/3, 350/35 ng/mL, respectively. The IS concentration was 12.5 ng/mL. One sample at each concentration was prepared and injected for 6 times. Six different lots of plasma were used to prepare set 2 and set 3 validation samples. Set 2 samples were prepared by extracting blank plasma then spiking FDB/CFB and IS into the extracted matrix at the same concentration as set 1. Set 3 were prepared by spiking FDB/CFB at 6/0.6, 60/6, and 700/70 ng/mL in 6 different lots of plasma and extracting the samples as described in sample preparation section. Samples were injected in the order of set 1, 2, and 3 for each of the 6 lots of matrices for low concentration, followed by medium and high concentrations in the same injection order. The data from set 1 and set 2 were used to define overall system and detector performance, absolute and relative matrix effects, results from set 3 defined recovery and overall process efficiency.

The stability of FDB and CFB was evaluated in the following conditions: 3-freeze-thaw cycles, freezer (−70 °C), room temperature (22–25 °C) in plasma for 72 h and injection solvent (in autosampler vial) for 24 h and 96 h. Stock solution of FDB was tested for 2 months at −70 °C. IS working solution was tested at room temperature (22–25 °C) for 8 days. The treated samples were measured in triplicate at low and high concentrations and compared to the corresponding untreated samples. Stability was expressed as % remained and calculated as follows: % remained = $100 \times C_{treated}/C_{untreated}$, where *C* represents the measured mean concentration.