Journal of Chromatography B, 877 (2009) 1040–1046

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Development of a sensitive and selective LC-MS/MS method for the determination of α-fluoro-β-alanine, 5-fluorouracil and capecitabine in human plasma

Hermes Licea-Perez*, Sherry Wang, Chester Bowen

Worldwide Bioanalysis, Drug Metabolism and Pharmacokinetics, GlaxoSmithkline Pharmaceuticals, 709 Swedeland Road, King of Prussia, PA 19406, USA

ARTICLE INFO

Article history:
Received 1 October 2008
Accepted 7 February 2009
Available online 13 February 2009

Keywords:
α-Fluoro-β-alanine (FBAL)
5-Fluorouracil (5-FU)
Capecitabine
Derivatization
Dansyl chloride
Mass spectrometry

ABSTRACT

A sensitive and selective quantitative method to determine α-fluoro-β-alanine (FBAL), 5-fluorouracil (5-FU), and capecitabine (Cape) from a single human plasma aliquot (50 μL) has been developed and validated. First, 5-FU and Cape were extracted by liquid–liquid extraction (LLE) using a mixture of acetonitrile and ethyl acetate. This was followed by derivatization with dansyl chloride. The dansyl-derivatives from 5-FU and Cape were further purified using LLE with methyl tertiary-butyl ether (MTBE) and analyzed using a reversed-phase analytical column "Primesep D" (2.1 mm × 50 mm; 5 μm) with embedded basic ion-pairing groups. The remaining aqueous phase containing FBAL was treated with dansyl chloride and the dansyl-FBAL was purified by solid phase extraction. Ultra high pressure liquid chromatography (UPLC) technology on a BEH C18 stationary phase column with 1.7 μm particle size was used for analysis of dansyl-FBAL. The method was validated over the concentration ranges of 10–10,000, 5–5000, and 1–1000 ng/mL for FBAL, 5-FU, and Cape, respectively. The results from assay validation show that the method is rugged, precise, accurate, and well suited to support pharmacokinetic studies where approximately 300 samples can be extracted and analyzed in 1 day.

1. Introduction

Capecitabine (Cape), a prodrug of 5-fluorouracil (5-FU), is a novel oral fluoropyrimidine carbamate that is preferentially metabolized to the cytotoxic moiety 5-FU in target tumor tissues [1,2]. Cape chemotherapy has been proven to stop cancer cells from growing and decrease the size of tumors in metastatic breast cancer and colorectal cancer [3]. In humans, Cape is rapidly and extensively absorbed from the gastrointestinal tract and then metabolized primarily in the liver by carboxylesterase to 5-deoxyfluorocytidine (5-DFCR) followed by deamination to 5-deoxyfluorouridine (5-DFUR) by cytidine deaminase [4,5]. In tumor cells, 5-DFUR is further converted to 5-FU by thymidine phosphorylase [4,5]. Subsequently, 5-FU is catabolized by dihydropyrimidine dehydrogenase (DPD) to dihydro-5-fluorouracil (FUH_2), 5-fluoroureidopropionic acid and α-fluoro-β-alanine (FBAL) [4,5]. Even though Cape is generally well tolerated, some patients can develop severe gastrointestinal toxicity which results in diarrhea [3]. Gastrointestinal toxicity is thought to be caused by the release of 5-DFUR within the small intestine. In addition, FBAL has been proven to induce cardiotoxicity and neurotoxicity in some patients [6,7]. Due to these issues, a sensitive and accurate bioanalytical method for the analysis of FBAL, 5-FU, and Cape is required for monitoring chemotherapy treatment in cancer patients.

Although several methods have been reported for analysis of Cape, 5-FU, and/or their pyrimidine metabolites in biological matrixes, only a few procedures include the analysis of FBAL. Due to the remarkable differences in polarity between Cape, 5-FU, and FBAL it has been a great challenge to develop a method for the simultaneous determination of all analytes in biological matrixes with the lower limit of quantification (LLQ) between 1 and 10 ng/mL. Analytical methods measuring different combinations of Cape with its metabolites such as; Cape with 5-FU, 5-DFCR, and 5-DFUR [8–10]; Cape with 5-DFCR, and 5-DFUR [11]; Cape with 5-FU [12]; 5-FU with FBAL [13,14], have been reported. Also, the bioanalysis of a single analyte has been reported for FBAL [15,16] and 5-FU [17]. The major limitation of previously reported analytical methods is the insufficient sample throughput, due to large volume of biological specimens or long retention times of the assay (UV-based methods). Dhananjeyan et al. [8] described a method for analysis of Cape, 5-DFCR, 5-DFUR, and 5-FU in mouse plasma using protein precipitation and HPLC–UV detection, however the LLQ of the assay was 1000 ng/mL and the run time was 10 min. Zufía et al. [9] developed a HPLC–UV method for a combination analysis of Cape, 5-DFUR, 5-FU, and FUH_2 in a single step extraction, but the

* Corresponding author. Tel.: +1 610 270 5116; fax: +1 610 270 5005.
E-mail address: hermes.2.licea_perez@gsk.com (H. Licea-Perez).

1570-0232/$ – see front matter © 2009 Published by Elsevier B.V.
doi:10.1016/j.jchromb.2009.02.025

throughput was limited due to the use of 500 μL of plasma and a run time that exceeded 30 min. Moreover, the LLQ achieved was only 25 ng/mL for Cape and its nucleoside metabolites. Guichard et al. [10] developed and validated a liquid chromatography tandem mass spectrometry (LC–MS/MS) method for the simultaneous analysis of Cape, DFCR, 5-DFUR, and 5-FU requiring only 50 μL of mouse plasma. However, the run time was 12 min and the LLQ for 5-FU was 50 ng/mL. Xu and Grem [11] reported a method employing direct plasma sample injection and liquid chromatography mass spectrometric detection (LC–MS) for simultaneous determination of Cape, 5-DFCR, and 5-DFUR, however the method was not suitable for analysis of FBAL or 5-FU, and the run time exceeded 20 min.

To our knowledge, there is no published method describing the analysis of Cape, 5-FU, and FBAL in a single human plasma aliquot with a level of sensitivity in the range 1–10 ng/mL. In this paper, we describe a relatively high-throughput LC-MS/MS method for the determination of Cape, 5-FU, and FBAL from single plasma aliquot (50 μL) in a 96-well plate format. Due to the remarkable differences in polarity among the three analytes and to difficulties finding an analytical column to retain and separate them, the method employs a novel derivatization procedure with dansyl chloride.

2. Experimental

2.1. Chemicals and reagents

5-FU, [$^{13}C_1$,$^{15}N_2$]-FU, Cape, [$^{2}H_{11}$]-Cape, 5-DFCR, 5-DFUR, and [$^{13}C_3$]-FBAL were purchased from Toronto Research Chemicals (North York, ON, Canada). FBAL was purchased from ABCR GmbH & Co (Karlsruhe, Germany). Dansyl chloride was purchased from MP Biomedicals (Solon, OH, USA). HPLC grade acetone, acetonitrile, ethyl acetate, and methanol were purchased from Burdick and Jackson (Muskegon, MI, USA). Methyl tertiary-butyl ether (MTBE) and formic acid were purchased from EMD Scientific (Gibbstown, NJ, USA). Human plasma was obtained from Bioreclamation Inc. (East Meadow, NY, USA).

2.2. Equipment

An Eppendorf 5810R centrifuge with a rotor capacity for four 96-well plates (Brinkmann Instrument, Westbury, NY, USA), and a Mettler UMX2 balance (Columbus, OH, USA) were used. A Hamilton Mircrolab STAR liquid handler (Reno, NV, USA) was used for plasma transfer and a TomTec Quadra 3 SPE (Hamden, CT, USA) was used for liquid transfer. Arctic White LLC 96-well round 2 mL plates with silicone and PTFE film seal mats (Bethlehem, PA, USA) were used to extract analytes and their internal standards from plasma. One milliliter deactivated (silanized) glass vials along with 96-well plate covers (Blue CapMat with Pre-Cut T/S Septa) from MicroLiter Analytical Supplies (Suwanee, GA, USA) were used for sample introduction to the LC–MS/MS. The SPE sample clean-up after derivatization of dansyl-FBAL was carried out on the Oasis 10 mg Oasis MAX 96-well plates from Waters (Milford, MA, USA).

2.3. Preparation of standards and quality control (QC)

Stock solutions of FBAL, 5-FU, Cape, [$^{13}C_1$,$^{15}N_2$]-FU, [$^{2}H_{11}$]-Cape, 5-DFCR, and [$^{13}C_3$]-FBAL were individually prepared in water at concentrations of 1.0 mg/mL and stored at 4 °C. Appropriate volumes of stock solutions of FBAL, 5-FU, and Cape were combined and diluted with water to make the working solution for calibration standards (WS) and quality control (QC) samples (WQ) containing FBAL/5-FU/Cape at 200/100/20 μg/mL. The percentage of non-matrix solvent used to spike calibration standards and QC samples was less than 5% (v/v). The WS was used to make calibration standards at 10000/5000/1000, 5000/2500/500, 1000/500/100, 500/250/50, 100/50/10, 50/25/5, 20/10/2 and 10/5/1 ng/mL of FBAL/5-FU/Cape using a serial dilution procedure. The WQ was used to make QC samples at 50000/25000/5000, 10000/5000/1000, 8000/4000/800, 500/250/50, 40/20/4, and 10/5/1 ng/mL of FBAL/5-FU/Cape. QC samples were divided into 0.5 mL aliquots and frozen at −20 °C or extracted immediately. In the first validation run, freshly prepared QC samples were analyzed against freshly prepared calibration standards. For each subsequent validation run, frozen replicate aliquots of the QC samples were thawed at room temperature and analyzed against a freshly prepared standard curve.

2.4. Sample extraction

MTBE (1 mL) was added to each well of the 2 mL ArcticWhite 96-well polypropylene plate. The plate was sealed with the ArctiSeal mat and vortex mixed in an inverted position for 3 min. Subsequently, the MTBE was discarded and the plate was left to dry in the chemical hood. This wash step was used to remove any plastic residue from the plates and plate seals. Plasma samples (50 μL) then were transferred to the washed 96-well plate using a Hamilton STAR liquid handler. A 300 μL aliquot of internal standard solution in acetonitrile (25/100/50 ng/mL of [$^{13}C_3$]-FBAL/[$^{13}C_1$,$^{15}N_2$]-FU/[$^{2}H_{11}$]-Cape) was added to all wells with the exception of the blanks, which received 300 μL of acetonitrile. Following the addition of 0.8 mL of ethyl acetate, the wells were capped with the ArctiSeal mat; vortex mixed for 3 min, and centrifuged at 3220 × *g* for 5 min. The organic layer was used for analysis of 5-FU and Cape (see Section 2.4.1) while the remaining aqueous phase was used for analysis of FBAL (see Section 2.4.2).

2.4.1. Extraction of 5-FU/Cape

The ethyl acetate/acetonitrile layer containing 5-FU/Cape, was transferred to another washed ArcticWhite 96-well 2 mL polypropylene 96-well plate using a TomTec liquid handler and the solvent evaporated under a stream of nitrogen at 45 °C. After evaporation, 100 μL of 10 mg/mL dansyl chloride in acetonitrile and 100 μL sodium bicarbonate (100 mM; pH 11), were added to all wells. The plate was sealed and vortex mixed for 3 min, followed by incubation at 60 °C for 30 min. After incubation, 1 mL of MTBE was added to all wells to extract the dansyl-derivatives of 5-FU and Cape. After vortex-mixing and centrifugation, the MTBE layer was transferred to a polypropylene 96-well plate containing 1 mL deactivated (silanized) glass-inserts using a TomTec liquid handler and evaporated to dryness under a stream of nitrogen at 45 °C. The extract then was reconstituted in 100 μL of 30/70 (v/v) acetonitrile:water.

2.4.2. Extraction of FBAL

The remaining aqueous phase from section 2.4 containing FBAL was incubated with 100 μL of dansyl chloride (10 mg/mL) in acetone and 100 μL sodium bicarbonate (100 mM; pH 11) at 60 °C for 5 min. Following addition of 0.5 mL of water, the wells were capped with the ArctiSeal mat; vortex mixed for 3 min, and centrifuged at 3220 × *g* for 5 min. Three hundred microliters of the supernatant then were loaded onto a 10 mg Oasis MAX, 30 μm, SPE 96-well plate which was previously conditioned with 1 mL methanol and 1 mL water. The loaded SPE plate was then washed with 1 mL of water, 1 mL of methanol. Dansyl-FBAL then was eluted twice with 250 μL volumes of 2% formic acid in methanol (freshly prepared) into a 2 mL 96-well plate containing 1 mL deactivated (silanized) glass-inserts. These sample extracts were evaporated to dryness at 45 °C under a stream of nitrogen, reconstituted with 500 μL of 30/70 acetonitrile/water, capped and vortex mixed and centrifuged before analysis.