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Short communication

Validation of an isocratic HPLC method to detect 2-fluoro-β-alanine for the analysis of dihydropyrimidine dehydrogenase activity

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ABSTRACT

The efficacy of the chemotherapeutic drug 5′-fluorouracil is reduced by catabolism to 2′-fluoro-β-alanine (FBAL), a three-step reaction in which dihydropyrimidine dehydrogenase (DPD) catalyzes the rate-limiting step. To study *in vitro* DPD activity, we developed and validated an isocratic, reverse-phase HPLC method to detect and quantify FBAL without using multiple columns or radiolabeled substrates. Pre-column derivatization of FBAL was performed using *o*-phthalaldehyde in the presence of two sulfur donors, ethanthiol or β-mercaptoethanol, and the resulting products assayed. Calibration curves were linear over a range of 10–200 μg/ml and the method was successfully applied to the examination of DPD activity in cultured cells.

1. Introduction

Systemic administration of the chemotherapeutic drug 5′-fluorouracil (5FU), commonly used to treat colorectal, breast, and head-and-neck cancers, can cause severe patient side effects, including gastrointestinal distress, myelosuppression, and cardiotoxicity [1,2]. Alternatively, administration of a non-toxic 5FU prodrug coupled with tumor-specific expression of a prodrug-activating enzyme may ameliorate toxicity; this treatment strategy is termed suicide gene therapy. The enzyme/prodrug system bacterial cytosine deaminase/5′-fluorocytosine (bCD/5FC) has been widely studied for use in suicide gene therapy. Following transfection with the bCD enzyme, tumor cells become 5FC sensitive while CD deficient mammalian cells remain relatively 5FC resistant [3,4].

Expression of the endogenous mammalian enzyme dihydropyrimidine dehydrogenase (DPD; EC 1.3.1.2) reduces the effectiveness of the bCD/5FC system. DPD catalyzes 5FU catabolism to dihydrofluorouracil, which is further metabolized to 2-fluoro-β-alanine (FBAL), a compound readily excreted in patient urine (Fig. 1). Reportedly, normal DPD activity catabolizes >80% of systemically administered 5FU while DPD over-expression is associated with reduced 5FU serum exposure, half-life and efficacy, and increased patient mortality [5–7]. We predict that expression of another enzyme, bacterial uracil phosphoribosyltransferase (bUPRT) will reduce DPD-mediated 5FU catabolism because bUPRT catalyzes 5FU anabolism to cytotoxic products [8,9]. Theoretically, bUPRT expression with bCD will lower the intracellular 5FU concentrations available as substrate for DPD, thus decreasing FBAL formation (Fig. 1).

To study the role of DPD in 5FC metabolism, we developed a simple, reverse-phase high-performance liquid chromatography (HPLC) method to resolve *o*-phthalaldehyde (OPA) derivatives of FBAL from the structurally similar endogenous amino acids L-alanine and β-alanine. Unlike previously reported methods describing the separation of these compounds, this method is isocratic, utilizes a single column, and does not rely on radiolabeled substrates to achieve sensitive and reliable FBAL detection [10–15]. Reports indicate that OPA-derivatives of alanine and β-alanine are more stable at room temperature when derivatization reactions are carried out using ethanthiol (ET) as the sulfur donor instead of the more commonly used β-mercaptoethanol (MCE) [16,17]. Similar stability studies of the FBAL-OPA-derivatives have not been reported. Therefore, we compared FBAL stability when OPA derivatization was performed in the presence of ET or MCE. Here we report the establishment of a novel HPLC method for FBAL detection and compare results from two different derivatization methods.

2. Materials and methods

2.1. Chemicals and reagents

DL-2-Fluoro-β-alanine (FBAL) was purchased from Indofine (Hillsborough, NJ, USA). 5FU, L-alanine, MCE, and OPA were pur-

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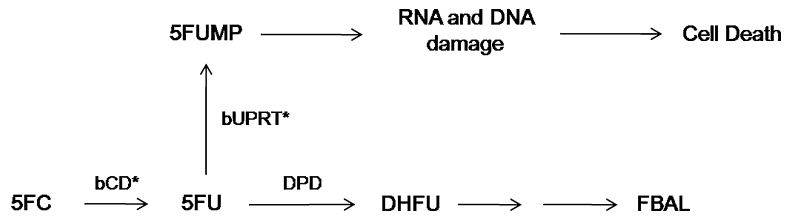

Fig. 1. 5FC activation pathway in mammalian cells transfected with *E. coli* enzymes (denoted by *). bCD, bacterial cytosine deaminase; bUPRT, bacterial uracil phosphoribosyltransferase; DPD, dihydropyrimidine dehydrogenase; 5FC, 5-fluorocytosine; 5FU, 5-fluorouracil; 5FUMP, 5-fluorouridine monophosphate; DHFU, dihydrofluorouracil; FBAL, 2-fluoro-β-alanine.

chased from Sigma–Aldrich (St. Louis, MO, USA). ET and β-alanine were purchased from Alfa Aesar (Ward Hill, MA, USA). Pterostilbene was a gift from the Sabinsa Corporation (Piscataway, NJ, USA). Potassium borate was purchased from Pfaltz & Bauer, Inc. (Waterbury, CT, USA). HPLC-grade water, acetonitrile, methanol, and phosphoric acid were purchased from JT Baker (Phillipsburg, NJ, USA). McCoy's medium was purchased from Sigma–Aldrich (St. Louis, MO, USA), prepared according to the manufacturer's instructions, and filter sterilized using a 0.22 μm Steritop® bottle top filter (Millipore Corporation, Billerica, MA, USA).

2.2. Preparation of stock and standard solutions

Stock solutions of alanine, β-alanine, and FBAL were prepared in HPLC-grade methanol to a final concentration of 200 μg/ml. Pterostilbene, used as an internal standard (I.S.), was prepared at a concentration of 100 μg/ml in HPLC-grade methanol. These solutions were wrapped in aluminum foil to protect from light and stored at −20 °C. OPA stock solution (designated methanolic OPA) was prepared in HPLC-grade methanol to a final concentration of 26 M prior to derivatization reactions.

2.3. Derivatization procedure

The buffer solution was prepared by dissolving potassium borate crystals in HPLC-grade water to a final concentration of 0.6 M, pH = 10. Derivatization reactions were carried out as follows:

(a) 15 μl MCE was added to 2.5 ml buffer solution and mixed with 0.5 ml methanolic OPA. OPA-MCE was added to amino acid solutions at 50% (v/v).
(b) 52 μl ET was added to 2 ml buffer solution, mixed with 0.63 ml methanolic OPA, and brought to a final volume of 10 ml with methanol. OPA-ET was added to amino acid solutions at 80% (v/v).

The derivatizing agents were stored at room temperature and discarded after 48 h.

2.4. Instrumentation and chromatographic conditions

The HPLC system consisted of a Shimadzu LC-20AD pump, SIL-10AD*VP* auto-injector, SCL-10A system controller, and SPD-10AV*VP* detector (Kyoko, Japan). Data collection and integration were performed using Shimadzu EZ Start 7.1.1SP1 software (Kyoto, Japan). The OPA-derivatives of L-alanine, β-alanine, and FBAL were separated under isocratic conditions on a Phenomenex Luna® C_{18} (2) analytical column (250 mm × 4.6 mm, 5 μm particle size) outfitted with a Phenomenex Security Guard Analytical Guard Cartridge System®. Flow rate was 0.6 ml/min. Mobile phase consisted of 65% MeOH, 15% ACN, 19.9% H_2O, 0.1% H_3PO_4 (v/v) and was filtered and degassed prior to use. Derivatized products were detected at ultraviolet wavelength of 334 nm and identified by retention times as compared to commercial standards.

The liquid chromatography–mass spectrometry-electrospray ionization (LC–MS-ESI) system was a Shimadzu LCMS-2010 EV (Kyoto, Japan) with the LC portion consisting of two LC-10AD pumps, a SIL-10AF*VP* auto-injector, a SPD-10A*VP* UV detector, and a SCL-10A*VP* system controller. Data analyses were performed using Shimadzu LC–MS LabSolutions Version 3 software (Kyoto, Japan). The chromatographic methods were identical to those employed in the HPLC methodology except that H_3PO_4 in the mobile phase was replaced by formic acid at 0.1% total volume. The mass spectrometer conditions consisted of a curved desolvation line (CDL) and a heat block temperature of 200 °C. The CDL, interface, and detector voltages were −10.0 V, 4.5 kV, and 1.5 kV, respectively. Vacuum was maintained by an Edwards® E2M30 rotary vacuum pump (Edwards, UK). Liquid nitrogen was used as a source of nebulizer gas (1.5 l/min).

2.5. Validation criteria

2.5.1. Sample preparation in McCoy's medium

Standard solutions were prepared fresh from stock solutions. The I.S. pterostilbene (100 μl) and McCoy's medium (100 μl) were added to each sample and vortexed for 30 s. Samples were dried to completion, reconstituted in 100 μl mobile phase, and derivatized as described in Section 2.3.

2.5.2. Linearity

A six-point standard curve of FBAL was generated by diluting FBAL stock solution in HPLC-grade water to final concentrations of 5, 10, 25, 50, 100, and 200 μg/ml. I.S. (100 μl) was added to each sample. The peak-area-ratios (PAR) of FBAL to I.S. were plotted against theoretical FBAL concentrations and linearity calculated using unweighted least squares linear regression analysis.

2.5.3. Accuracy, precision, and recovery

The intra-day (n = 8) and inter-day (n = 6) accuracy and precision of replicate assays were tested using the six-point standard curves. Accuracy was estimated based on the mean percentage error of measured concentration to actual concentration (Bias) and precision by the relative standard deviation (RSD).

2.5.4. Stability of FBAL and derivatives

Low and high concentrations (10 and 200 μg/ml, respectively) of FBAL derivatized with OPA-ET or OPA-MCE were assessed for stability following a 24 h incubation at ambient temperature (n = 3). FBAL and I.S. standards were prepared in McCoy's medium, dried, and reconstituted in the mobile phase. Derivatization reactions were carried out as described in Section 2.3 and 100 μl of sample was injected at 0 h. The solution remained at ambient temperature in the auto-injector followed by subsequent injections at various time points up to 24 h. Freeze–thaw stability was evaluated for FBAL at concentrations of 10 and 200 μg/ml (n = 3) according to guidelines for method validation [18]. Following the addition of I.S. and McCoy's medium (100 μl each), samples were dried and reconstituted in 200 μl mobile phase. An aliquot (100 μl) was derivatized

with OPA-ET and injected. A second aliquot (100 μl) was subjected to three freeze–thaw cycles (−80 °C) followed by derivatization and injection into the HPLC system.

2.6. Evaluation of FBAL formation

HCT116 human colorectal cancer cells (ATCC) were maintained at 37 °C and 5% CO_2 in McCoy's medium supplemented with 10% FBS. Cells were stably transfected with the mammalian expression vector pcDNA6 carrying genes encoding the enzymes bCD or bCD/UPRT. Transfection was performed using FuGene6 Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to manufacturers' recommendations. Positive selection and maintenance of transfectants was accomplished by culturing cells in medium supplemented with 6 μg/ml blasticidin. Expression of suicide enzymes and DPD were confirmed by immunoblot analysis (data not shown).

Stably transfected cells were seeded in 6-well plates at 2×10^5 cells/well in 2 ml media and incubated with 5FC (645 μg/ml). After 24 h incubation, 200 μl of acetonitrile:acetic acid (94:6, v/v) was added to each well. Cells were lysed via freeze–thaw and debris removed by centrifugation. I.S. (100 μg/ml) was added and samples were dried, reconstituted in mobile phase, and derivatized with OPA-ET as described in Section 2.3. Following filtration through a 0.2 μm membrane syringe filter (Pall Corporation, Ann Arbor, MI, USA), 100 μl of the supernatant was injected into the HPLC system.

3. Results and discussion

3.1. Derivatization, stability, and specificity

Derivatization reactions of FBAL were carried out as described in Section 2.3 and the identity of the products was confirmed using commercially purchased standards and by LC–MS analysis. The expected *m*/*z* ratios of the OPA-ET derivatives in the electropositive ion mode were calculated as formula weight of the compounds (FBAL, 107.1; L-alanine and β-alanine, 89.1; pterostilbene, 256.3) plus OPA-ET (180.3). The schematic reaction sequence for the OPA-ET derivatized FBAL is outlined in Fig. 2. The stability of the FBAL derivatives (10 and 200 μg/ml) was analyzed at ambient temperature. Mean recovery ($n = 3$) of OPA-ET derivatives was >88% ± 3 up to 24 h at both concentrations examined. Recovery of OPA-MCE derivatives was poor as the compound was not detectable after 3 h. Similar results were obtained in stability analyses of OPA-ET and OPA-MCE derivatives of L-alanine and β-alanine [16,17].

The developed HPLC method resulted in the following retention times of OPA-ET derivatives: FBAL-10 min and I.S.-7.8 min. When the analyte standards were run in McCoy's medium, no interfering peak was noted for the FBAL derivative. A minor peak eluted with I.S. and constituted <5%of the total area of the I.S. Background did not interfere with quantification (Fig. 3). The peaks corresponding with the OPA-ET derivatives of L-alanine and β-alanine eluted at 9 min. As FBAL quantification was the focus of this study, resolution of the L-alanine and β-alanine peaks was not pursued. The mean extraction efficiency of FBAL from media was >95% at 10 and 200 μg/ml ($n = 5$).

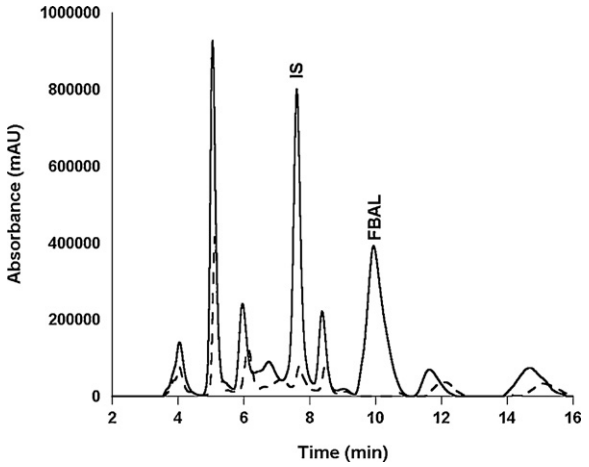

Fig. 3. Representative chromatogram illustrating separation of OPA-ET derivatives of FBAL and I.S. No interfering peaks between FBAL (solid line) and McCoy's medium (dashed line) were observed at a 0.6 ml/min flow rate. A minor peak eluted with the I.S. but did not interfere with quantification.

3.2. Linearity and lower limits of quantification (LLOQ) and detection (LLOD)

Standard curves of FBAL were linear over a range of 10–200 μg/ml ($R^2 \geq 0.991$) as determined by comparing PAR values of FBAL to the I.S. A typical mean regression curve is described by FBAL (μg/ml) = 0.005 ± 0.001*x* + 0.1 ± 0.05. The LLOQ was 10 μg/ml and the LLOD was 5 μg/ml for this method using a 100 μl sample. The concentration of quality control samples calculated using linear regression was within the accepted validation criteria [18].

3.3. Accuracy, precision, and recovery

Accuracy and precision of the method were evaluated over a range of FBAL concentrations from 10 to 200 μg/ml. The RSD and bias for intra-day ($n = 8$) and inter-day ($n = 6$) replicates (Table 1) is within the accepted validation criteria [18].

3.4. Stability of FBAL

Stability of FBAL ($n = 3$) following three freeze–thaw cycles was excellent with a mean recovery of 103 ± 19% at 10 μg/ml and 107 ± 24% at 200 μg/ml.

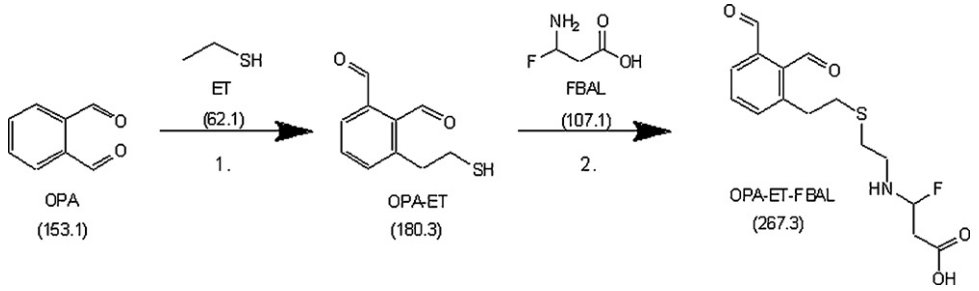

Fig. 2. Proposed reaction mechanism of FBAL derivatization. The product of reaction 1 is the derivatizing agent OPA-ET, and of reaction 2 is OPA-ET-FBAL. Molecular weights values are noted in parentheses below each compound.

Table 1
Standard curve parameter summary and mean back-calculated calibration curve concentrations and standard deviations (SD) for intra-day ($n = 8$) and inter-day ($n = 6$) runs.

FBAL (μg/ml)	Within			Between		
	Mean ± SD	RSD (%)	Bias (%)	Mean ± SD	RSD (%)	Bias (%)
10	11.6 ± 2.1	18.1	15.6	11.0 ± 1.7	15.3	9.7
25	23.9 ± 2.6	10.9	−4.5	22.1 ± 1.3	5.7	−11.7
50	49.5 ± 6.6	13.3	−1.1	48.6 ± 4.7	9.6	−2.8
100	104.0 ± 9.3	8.9	4.0	101.7 ± 4.2	4.2	1.7
200	198.8 ± 4.3	2.2	−0.6	199.0 ± 1.7	0.9	−0.5

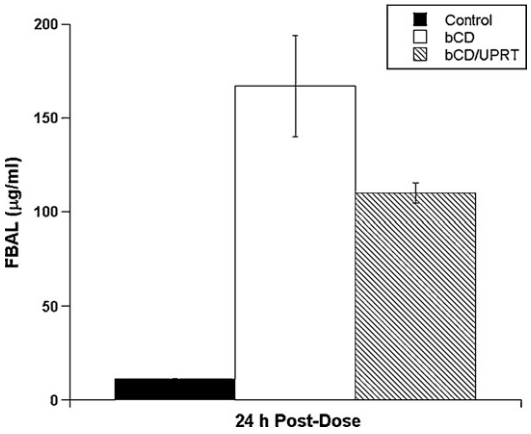

Fig. 4. Evaluation of DPD activity in mammalian cells. Concentrations (μg/ml) of FBAL detected in lysates of HCT116 cells expressing bCD or bCD/UPRT enzymes and control cells. Data illustrate metabolite levels following 24 h incubation with 5FC.

3.5. Evaluation of FBAL production

We applied the HPLC method to assess *in vitro* DPD activity by examining intracellular conversion of 5FC to FBAL in HCT116 cells expressing the suicide enzymes bCD or bCD/UPRT, as described in Section 2.6 ($n = 3$). FBAL was detected in cells expressing both bCD or bCD/UPRT 24 h post-dose, demonstrating applicability of this method (Fig. 4). FBAL concentrations in cells expressing bCD/UPRT were slightly lower as compared to bCD expressing cells, suggesting that bUPRT may partially limit DPD-mediated catabolism of 5FU, at least initially. Little FBAL (11 μg/ml) was detected in control cells. Since these cells lack an enzyme to metabolize 5FC, the levels detected were most likely a result of spontaneous deamination of 5FC to 5FU followed by conversion to FBAL by DPD. However, since the FBAL levels detected in control cells were significantly lower than those detected in enzyme-expressing cells, they do not interfere with accurate FBAL detection or quantification in our experiments.

4. Conclusions

In summary, we developed a reverse-phase, isocratic HPLC method to rapidly (<12 min) separate and quantify FBAL. Detection of OPA-ET derivatives of FBAL was accurate and precise over a concentration range of 10–200 μg/ml. Stability of OPA-ET derivatives was excellent at room temperature for up to 24 h whereas OPA-MCE derivatives quickly degraded. FBAL was stable following freeze–thaw cycles. This method was successfully utilized to examine *in vitro* activity of DPD. To our knowledge, this is the first report comparing stability of FBAL following OPA derivatization in the presence two different sulfur donors. Unlike previously reported methods, FBAL detection was achieved without use of multiple columns, mobile phase gradients, or radiolabeled substrates, making this a simple, rapid, and convenient assay for *in vitro* applications of suicide gene therapy. This method can be used in conjunction with our previously published method [19] for quantification of 5FC and its four major anabolic metabolites to enable a comprehensive analysis of *in vitro* 5FC metabolism. Additionally, this method may be further refined for *in vivo* pre-clinical and clinical studies or for use as a diagnostic tool for assessing *in vivo* DPD activity.

Acknowledgements

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