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Liquid chromatography method for the quantitation of the breast cancer resistance protein ABCG2 inhibitor fumitremorgin C and its chemical analogues in mouse plasma and tissues

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Abstract

Fumitremorgin C (FTC) was recently discovered to be a potent and selective inhibitor of the breast cancer resistance protein (BCRP/ABCG2). FTC was shown to reverse multidrug resistance mediated by BCRP and to increase the cytotoxicity of several anticancer agents in vitro. To support in vivo studies a reverse phase HPLC method with ultraviolet detection was developed to quantitate FTC in mouse plasma and tissues. Further, assay method validation was performed for the determination of FTC in mouse plasma. Plasma standard curves ranged from 0.03 to 30 μg/ml, while the various tissue assay ranges differed to some extent. The sample preparation consisted of acetonitrile precipitation with separation accomplished with a C18 Novapak column and a C18 pre-column utilizing an isocratic mobile phase of ammonium acetate and acetonitrile. UV detection was set at 225 nm for FTC and at 312 nm for roquefortine, the internal standard. The retention times were approximately 9.5 min for FTC and 13.0 min for roquefortine. The recoveries for FTC and roquefortine from plasma were 90.8 ± 5.8% and 111.6 ± 13.6%, respectively. The reported assay can be used for future study of BCRP resistance in vivo in different biological matrices. Further, we found that a more potent analogue of FTC, Ko143, was able to be extracted and detected, with a maximal UV absorbance at 320 nm under the conditions reported.

Keywords: Fumitremorgin; Breast cancer resistance protein

1. Introduction

Fumitremorgin C (FTC) is a mycotoxin recently discovered to be a specific and selective inhibitor of the breast cancer resistance protein (BCRP/ABCG2) [1–3]. The BCRP inhibitory activity of FTC was discovered when Rabindran and Greenberger tested a library of extracts from a variety of microorganisms in a cell-based transport screen. One of the extracts from the medium of *Aspergillus fumigatus* was found to be active and increased the cytoxicity of mitoxantrone in a BCRP overexpressing cell line. When studied further, the pharmacologically active ingredient in the extract was found to be the diketopiperazine FTC (Fig. 1A) [1]. FTC is relatively non-toxic and is able to completely reverse resistance in cell lines with high levels of BCRP-mediated resistance at micromolar concentrations (1–5 μM). The toxicity previously shown with FTC was tremors in day-old cockerels when FTC was administered orally as an extract [4]. Recently, several analogues of FTC have been synthesized to improve BCRP specificity and selectivity while diminishing the potential for toxicity [5]. Among these analogues, Ko143 (Fig. 1C) was found to be a specific and selective inhibitor of FTC with little toxicity in mice.

It has become apparent that the breast cancer resistance protein is potentially an important mediator of multidrug resistance [6] and FTC, because of its potent and selective in-

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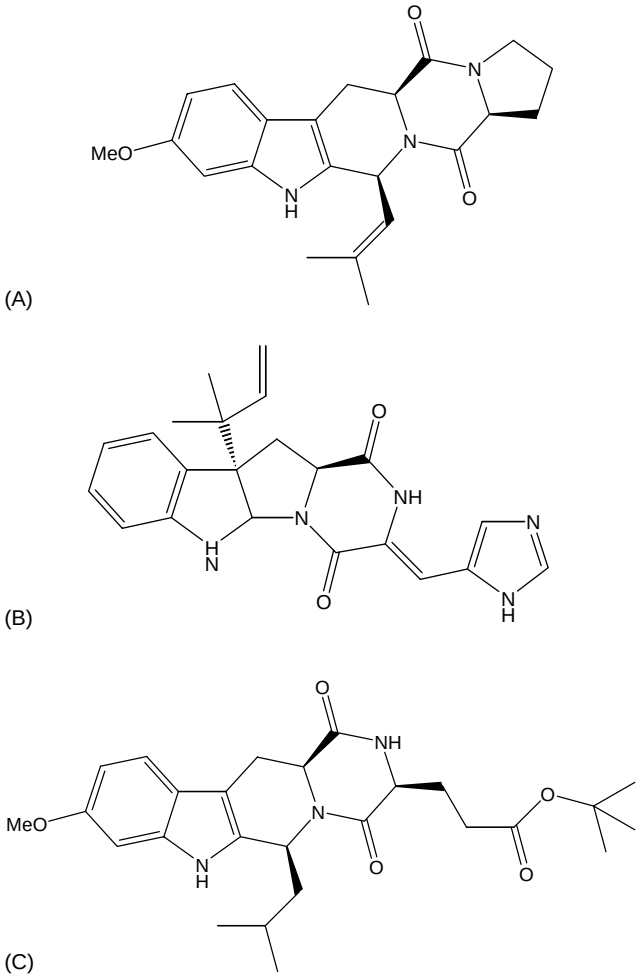

Fig. 1. The chemical structure of: (A) fumitremorgin C (FTC); (B) Roquefortine; and (C) Ko143.

hibition of BCRP may play a vital role in the study of the effects of BCRP on drug resistance and disposition. FTC and/or derivative compounds are also likely to be studied as modulators of BCRP resistance in vivo, and analogs may have the potential to be tested clinically. Hence, it is critical to have a simple and reliable method to assay FTC in cell systems and biological matrices to support those studies. FTC, because of it fungal origin, has been studied for sometime as a mycotoxin and cell cycle inhibitor [7,8]. FTC and other fumitremorgins have been isolated and extracted from fungal sources and detected using HPLC methods as a mixture [7], however this is the first report of a simple reverse phase HPLC method to determine FTC concentrations in biological matrices, specifically mouse plasma and tissues.

We have developed and validated a simple HPLC method for quantitation of FTC from mouse plasma using UV detection covering a broad range of concentrations (0.03–30 μg/ml). In addition, a method was developed to quantitate FTC in mouse tissues including: brain, lung, liver, heart, spleen, kidney, skeletal muscle and fat as well as the human ovarian Igrov1-derived T8 tumor grown as a xenograft in mice. The assay method described herein was developed initially to support an in vivo study following intravenous administration of FTC, 25 mg/kg, to tumor bearing mice. A structurally similar diketopieprazine, roquefortine was used as an internal standard for the assay described herein (Fig. 1B). Additionally, the assay was able to detect the recently synthesized Ko143 (Fig. 1C) analogue of FTC.

2. Experimental

2.1. Chemicals and reagents

FTC was a gift from Dr. Susan Bates, Medicine Branch, National Cancer Institute. Ko143 was obtained as a gift from Dr. Alfred Schinkel, Netherlands Cancer Institute. Roquefortine and ammonium acetate were purchased from Sigma Chemical Company (St. Louis, MO, USA), acetonitrile and methanol (HPLC grade) were obtained from B&J (Muskegon, MI, USA), blank mouse plasma was obtained from Valley Biomedical Products and Services (Winchester, VA, USA), and deionized water was obtained from a Hydro® Pico Pure System (Hydro Services and Supplies, Rockville, MD, USA).

Stock solutions of FTC were prepared by dissolution in absolute methanol at a concentration of 2 mg/mL and stored at −20 °C protected from light. Separate FTC stock solutions were prepared for calibration curves and quality controls. Ko143, provided as a solution in DMSO, was further diluted with methanol to a final concentration of 0.1 mg/mL. Roquefortine stock was prepared by dissolution in acetonitrile at a concentration of 1 mg/mL and stored at −20 °C and diluted just prior to use.

2.2. Chromatographic separation and detection

The HPLC system consisted of a Hewlett-Packard 1100 series liquid chromatograph equipped with a photodiode-array (2 nm resolution) detector controlled by HPLC 3D Chem Station software Rev A.03.03 using a Hewlett-Packard Vectra VL Pentium computer. The mobile phase consisted of an isocratic flow of 10 mM ammonium acetate buffer (pH 4.0) and acetonitrile in the ratio 70:30 pumped at a flow rate of 1 mL/min with a total run time of 20 min. The chromatographic separation of FTC was accomplished with a Waters Nova-Pak® C18 (3.9 × 150 mm) column with a 4 μM particle size (Milford, MA, USA) and a Phenomenex C18 (4 mm length × 3 mm i.d.) guard column cartridge system (Torrance CA, USA). UV absorbance detection was set at 225 nm for FTC and 312 nm for roquefortine. The optimal ultraviolet wavelength for the detection of the FTC analogue Ko143 was 320 nm. The absorbance spectra for FTC, roquefortine, and Ko143 are shown in Fig. 2.

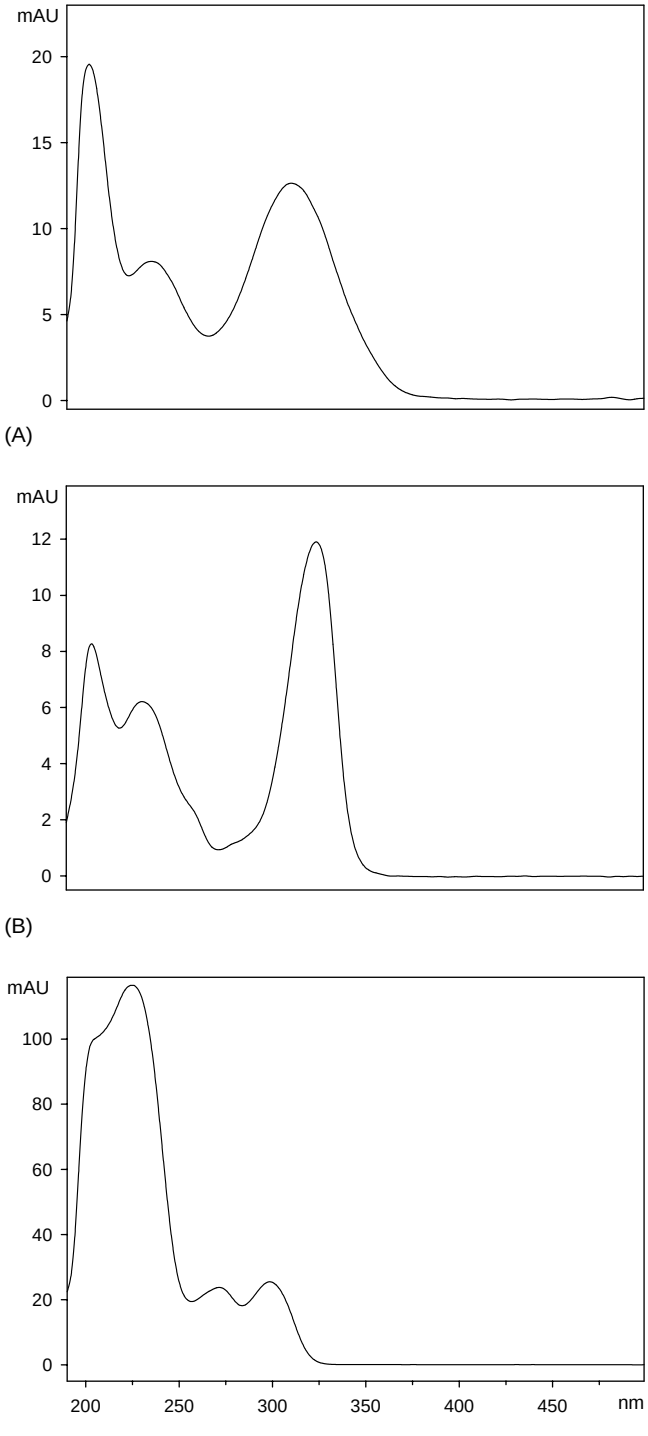

Fig. 2. Absorbance spectrum of: (A) fumitremorgin C (FTC) 10 μg/mL; (B) roquefortine 1 μg/mL; and (C) Ko143 10 μg/mL.

2.3. Standards, quality controls, and calibration curves

2.3.1. Plasma samples

Calibration curves and quality controls of FTC were prepared by adding small volumes of diluted stock solution to pooled mouse plasma. The standards were prepared at concentrations of 0.03, 0.1, 0.3, 1,3,10, and 30 μg/mL. The quality controls were prepared as 0.25, 2.5, and 25 μg/mL. All standards and quality controls were stored at −80 °C and assessed for freeze–thaw stability over three cycles.

2.3.2. Tissue samples

All tissues were dissected as whole organs from the mice. The Igrov1/T8 human ovarian cancer cells were obtained from Maliepaard et al. [9]. These cells, overexpressing BCRP, were created by selection of Igrov1 cells with topotecan. Tumor was obtained by growing the Igrov1/T8 cells as xenografts in the flanks of female SCID mice (5×10^6 cells per mouse). All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) (University of Maryland at Baltimore, and University of Pittsburgh).

The calibration curves and quality controls for the tissue were prepared by adding small volumes of FTC stock solutions to pooled blank tissue homogenates. Standards for brain and skeletal muscle were prepared at the following concentrations 0.0075, 0.015, 0.05, 0.15, 0.5, 1.5, 5 μg/gm with quality controls of 0.125, 1.25 μg/g. The standard curves for fat, heart, tumor, kidney and liver were prepared as 0.03, 0.1, 0.3, 1, 3, and 10 μg/gm with quality controls of 0.25 and 2.5 μg/gm. The standards for the lung were prepared as 0.06, 0.12, 0.4, 1.2, 4, 12, 40, and 120 μg/gm and for spleen as 0.02, 0.04, 0.13, 0.4, 1.3, 4, and 13.3 μg/gm while the quality controls were 0.3 and 3.3 μg/gm. The tissue concentration ranges represent those optimal to support the in vivo study described.

2.3.3. Plasma samples

A 100 μL plasma sample or standard was precipitated and deproteinized using 500 μL of a 60 ng/ml solution of roquefortine (internal standard) in acetonitrile. The samples were vortexed for 30 s and centrifuged at 19,000 × *g* at 4 °C for 10 min. The supernatant was removed and evaporated to dryness under a stream of nitrogen in a Multivap analytical evaporator (Organomation, South Berlin, MA, USA) at 35 ± 5 °C. The residue was then reconstituted with 100 μL of mobile phase solution (10 mM ammonium acetate (pH 4.0):acetonitrile, 70:30) and vortexed for 15 s and allowed to stand for 10 min. The reconstituted samples were transferred to a HPLC autosampler vial and 80 μL were injected into the HPLC system.

2.3.4. Tissue samples

A method based on the plasma extraction procedure was developed for the following tissues: brain, liver, kidney, spleen, lung, heart, skeletal muscle, and fat and T8 tumor. Tissue samples were prepared similarly to plasma samples except for the following differences. Following dilution with phosphate-buffered saline (PBS) (pH 7.4) in a ratio of 1:1 (w/v) for brain, tumor, lung, fat and kidney, 1:2 (w/v) for spleen, and 1:3 (w/v) for heart and skeletal muscle, tissues were homogenized in a Polytron Homogenizer (Brinkman

Instruments, Westbury, NY, USA). The various tissue to buffer ratios represented the optimal dilutions based on extraction efficiency and the experimental tissue concentrations found following administrations of 25 mg/kg FTC to tumor bearing mice. The tissue samples were prepared using a method similar to the plasma procedure; however the initial weight of the tissue processed was different. For tumor, liver, kidney, heart, and fat 100 mg of tissue was weighed and processed, for brain and skeletal muscle 200 mg of tissue was weighed and processed, for spleen 75 mg of tissue was weighed and processed and 25 mg of tissue was processed in the case of lung. Samples were precipitated and deproteinized using acetonitrile, containing roquefortine (60 ng/mL), at a volume five-fold greater than the homogenate volume.

2.4. Calibration, precision, and accuracy

The plasma assay was validated to conform to the recommendations for method validation as described in the FDA Guidance for Bioanalytical Method Validation [10]. The validation procedure consisted of four separate runs, with the third validation run also consisting of three freeze–thaw cycles of the quality controls, and the fourth run consisting of seven replicates of each quality control. Each validation run consisted of duplicate standards and triplicate quality controls for runs one through three and seven replicates for run number four.

Weighted least squares regression analysis was used to define the calibration curves. The regression analysis was performed using Sigma Plot 2000, version 6 using the equation $y = mx + b$, which was fitted to the data using a weight of $1/y^2$, where y represents the ratio of the FTC area to the roquefortine area, x the spiked FTC concentration, m the slope of the regression line and b the y-intercept.

The lower limit of quantitation (LLOQ) was determined to be the concentration yielding a signal-to-noise ratio of equal to or greater than 10, while the lower limit of detection (LOD) was defined as the concentration that yielded a signal-to-noise ratio of approximately 3.

Precision and accuracy of the inter- and intra-assay runs were calculated for the plasma calibration curves and quality control samples. Precision was calculated as the coefficient of variation (CV%) and the accuracy was calculated as the absolute % error.

2.5. Recovery and stability

To assess the extraction efficiency of FTC and roquefortine, extracted plasma samples were compared with unextracted mobile phase solutions in 10 mM ammonium acetate (pH 4) and acetonitrile (70:30) each sampled in triplicate. Long-term stability studies were performed at −80 °C by subjecting three aliquots ($n = 3$) of each of the three quality control concentrations in plasma to 10-month storage and comparing them with freshly prepared QC samples. An error of greater then 15% from the expected concentrations was considered unacceptable.

3. Results and discussion

3.1. Chromatographic separation and detection

The chromatographic conditions for plasma described above yielded retention times of 9.5 and 13 min for FTC and roquefortine respectively. Chromatograms resulting from the assay are depicted in Fig. 3. Fig. 3A shows the chromatogram from pooled blank mouse plasma and 3B shows the mouse plasma sample spiked with 3 μg/mL of FTC and 300 ng/mL of roquefortine. The lower limit of quantitation of the assay, 30 ng/mL yielded a signal-to-noise ratio of 11.6 ± 1.41 ($n = 6$). The lower limit of detection of FTC in mouse plasma was found to be 10 ng/mL, with a signal-to-noise ratio of 3.3.

An assay was also developed for each mouse tissue as previously described. The assays were linear within the concentration ranges of the assay. The retention times of FTC and roquefortine following tissue extraction ranged from 8.4 to 9.5 min and from 11.7 to 13.5 min, respectively. The tissue standards and samples were free of any major interfering peaks. Fig. 4 shows typical chromatograms of the tissue analysis. Fig. 4A shows the chromatogram obtained from blank pooled tumor and Fig. 4B shows the chromatogram obtained from spiking tumor with 3 μg/g of FTC and 300 ng/g of roquefortine with detection at 225 nm.

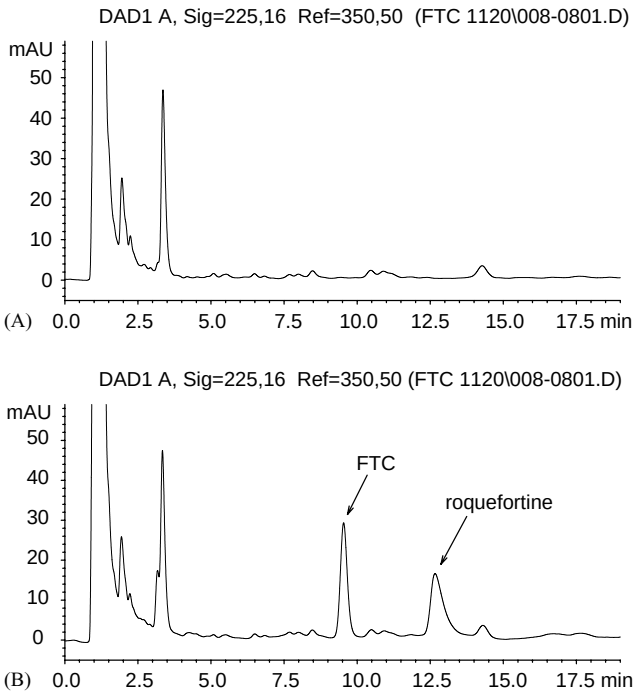

Fig. 3. Chromatogram of: (A) blank mouse plasma and (B) mouse plasma spiked with 3 μg/mL of fumitremorgin C (FTC) and 300 ng/ml (final concentration) of roquefortine. Absorbance wavelength 225 nm.

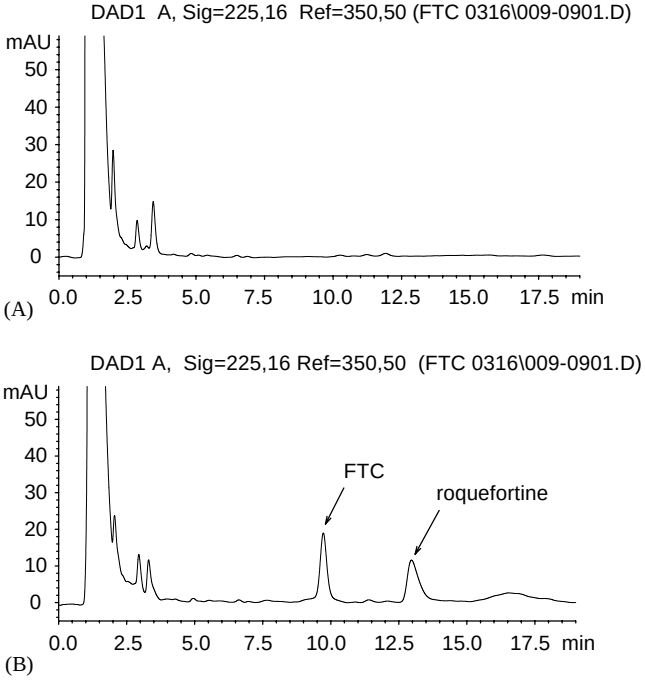

Fig. 4. Chromatogram of: (A) blank human Igrov1/T8 tumor (mouse xenograft) and (B) of human Igrov1/T8 tumor (mouse xenograft) spiked with 3 μg/g of fumitremorgin C (FTC) and 300 ng/g of roquefortine. Absorbance wavelength 225 nm.

This assay method was applied to detect the new BCRP inhibitor Ko143 in mouse plasma. The chromatogram obtained from plasma spiked with 10 μg/ml of Ko143 is shown in Fig. 5. Ko143 had a maximum absorbance at 320 nm (Fig. 2B) and a retention time of 4.5 min. The retention time of 4.5 min should allow for baseline resolution with the internal standard roquefortine (RT = 13 min).

3.2. Standards, quality controls, and calibration curves

The plasma HPLC assay was fully validated to conform to the guidelines set forth in the FDA Guidance on Bioanalytical Method Validation [10]. The plasma standard curve was linear throughout the concentration range assayed (0.03–30 μg/mL). The slope and intercept represent three

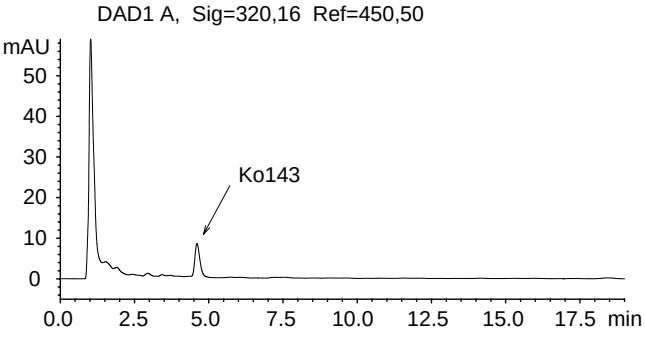

Fig. 5. Chromatogram of mouse plasma spiked with 10 μg/mL of Ko143. Absorbance wavelength 320 nm.

Table 1
Intra-assay precision and accuracy of FTC in mouse plasma

Spiked concentration ($n = 7$ per concentration) (μg/mL)	Mean found (μg/mL)	S.D. (ng/mL)	Precision (%CV)	Accuracy (% error)
0.25	0.242	0.017	7.11	−3.34
2.5	2.43	0.23	9.45	−2.62
25	24.0	2.5	10.3	−3.98

Table 2
Inter-assay precision and accuracy of FTC in mouse plasma

Spiked concentration ($n = 16$ per concentration) (μg/mL)	Mean found (μg/mL)	S.D. (ng/mL)	Precision (%CV)	Accuracy (% error)
0.25	0.240	0.016	6.6	−4.1
2.5	2.51	0.21	8.3	0.43
25	25.1	2.5	9.8	0.35

standard curves each containing duplicate standards run on 3 consecutive days. The slope ($m = 0.35188 \pm 0.0324$ for the plasma calibration curve $n = 3$, mean ± S.D.) and y-intercept ($b = 0.02848 \pm 0.004117$ for the plasma calibration curve $n = 3$, mean ± S.D.) were calculated for each calibration curve. The correlation coefficient (r^2) was 0.99188 ± 0.00569 for the plasma calibration curves $n = 3$, mean ± standard deviation. The accuracy for each calibration point, other than the LLOQ, was within 15% of the expected value, and was within 20% of the expected value at the LLOQ [10]. The intra- and inter-assay precision and accuracy for the plasma FTC assay are presented in Tables 1 and 2.

3.3. Recovery and stability

The extraction efficiencies from plasma were 90.8 ± 5.8% for FTC and 111.6 ± 13.6% for roquefortine. Complete recovery with this method was obtained with less than 10% variability between unextracted and extracted plasma samples.

Long-term stability studies over a 10-month period indicated less than 10% loss for all samples studied. Evaluation of freeze thawing of each matrix revealed no significant change in the quantitation of FTC. The freeze thaw stability for plasma was assessed over three full freeze thaw cycles ($n = 3$) for plasma quality controls. For all the three quality control samples, there was less than 15% loss of FTC.

4. Conclusion

FTC and its analogues are probably the most potent and selective agents available to inhibit and reverse resistance caused by BCRP. At a concentration of 5 μM, FTC causes complete reversal of resistance mediated by BCRP in vitro, and at 1 μM it caused inhibition of the resistance and increased amounts of cytotoxic drugs retained by BCRP ex-

pressing cells [3]. The recent reports of new FTC related compounds that have been developed as BCRP inhibitors [11] make it very likely that FTC or these analogues would be used in an in vivo situation to study their effects on BCRP-mediated resistance and effects on drug uptake into tissues and tumors. Hence, there is a need to quantitate the levels of FTC and analogues thereof in biological matrices.

We have developed a simple and reliable HPLC method to quantitate FTC levels in mouse plasma and tissues and have also validated the plasma assay. The assay for plasma has a linear range of 0.03–30 μg/mL and was stable over three freeze thaw cycles and following storage for a 10-month period while the assay for the different tissues had a total range of 0.075–120 μg/g. Previously published studies report the effect of temperature, light and water activity on fumitremorgin production by *Neosartorya fischeri* [7], and separation of fumitremorgins and other mycotoxins from fungal extracts by HPLC [12]. In the study by Nielsen et al. [7], FTC was detected and separated from other fumitremorgins when extracted from fungal cultures using a C18 reverse phase column and a mobile phase of acetonitrile and water run as a gradient at a flow rate of 2 mL/min. However, the report does not describe the limit of quantitation or detection of FTC. Ours is the first study of chromatographic separation and quantitation of FTC in biological matrices. The described assay is also able to detect and quantitate the new FTC analog Ko143 in mouse plasma. In addition, this assay can be extrapolated to a clinical setting to study levels of FTC (or analogues) in human patients, should these compounds reach the clinical setting for use in combination with anticancer agents.

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