Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Chromatography B, 831 (2006) 147–155
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Quantitative determination of zebularine (NSC 309132), a DNA methyltransferase inhibitor, and three metabolites in murine plasma by high-performance liquid chromatography coupled with on-line radioactivity detection

Jan H. Beumer[a,*], Erin Joseph[a], Merrill J. Egorin[a,b,c], Joseph M. Covey[d], Julie L. Eiseman[a,c]

[a] *Molecular Therapeutics/Drug Discovery Program, University of Pittsburgh Cancer Institute, Pittsburgh, PA 15213, USA*
[b] *Department of Medicine, School of Medicine, University of Pittsburgh, Pittsburgh, PA 15213, USA*
[c] *Department of Pharmacology, School of Medicine, University of Pittsburgh, Pittsburgh, PA 15213, USA*
[d] *Toxicology and Pharmacology Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Rockville, MD 20852, USA*

Received 7 October 2005; accepted 30 November 2005
Available online 20 December 2005

Abstract

The metabolism of zebularine (NSC 309132), a novel agent that inhibits DNA methyltransferases, is still uncharacterized. To examine the in vivo metabolism of zebularine, an analytical method was developed and validated (based on FDA guidelines) to quantitate 2-[^{14}C]-zebularine and its major metabolites in murine plasma. Zebularine and its metabolites uridine, uracil and dihydrouracil were baseline-separated based on hydrophilic interaction chromatography by using an amino column. The assay was accurate and precise in the concentration ranges of 5.0–100 μg/mL for zebularine, 2.5–50 μg/mL for uridine, 1.0–10 μg/mL for uracil and 0.5–5.0 μg/mL for dihydrouracil. This assay is being used to quantitate zebularine and its metabolites in ongoing pharmacokinetic studies of zebularine.

Keywords: Zebularine; Metabolism; Murine; Radioactivity; DNA methyltransferase inhibitor

1. Introduction

The pyrimidine analogue zebularine (NSC 309132) (Fig. 1) is a novel anticancer agent originally investigated for its inhibitory effect on cytidine deaminase [1]. More recently, it has been shown to be a potent inhibitor of DNA methyltransferases [2], a class of enzymes involved in the epigenetic silencing of tumor suppressor genes [3,4]. Further, zebularine enhances the activity of decitabine, a clinically used DNA methyltransferase inhibitor, in both human and murine leukemia cell lines. Zebularine potentiates decitabine, most likely by inhibition of cytidine deaminase-facilitated degradation of decitabine, and through direct inhibition of DNA methyltransferases [5].

Based on in vitro and in vivo activity [6], zebularine has been proposed for clinical evaluation. In preparation for such clinical studies, plasma pharmacokinetics of zebularine have previously been characterized in mice, rats, and rhesus monkeys [7]. Recent in vitro studies indicated that zebularine is metabolized to uridine by aldehyde oxidase (EC 1.2.3.1) [8]. However, the in vivo metabolic fate of zebularine remains to be elucidated. The proposed metabolic scheme for zebularine is shown in Fig. 1. It includes: oxidation of zebularine to uridine by aldehyde oxidase; removal of the ribose moiety of zebularine and uridine by uridine phosphorylase (EC 2.4.2.3) to produce 2-pyrimidinone and uracil, respectively; reduction of uracil to dihydrouracil by dihydropyrimidine dehydrogenase (EC 1.3.1.2); and subsequent hydrolysis of dihydrouracil to car-

* Corresponding author. Tel.: +1 412 623 3238; fax: +1 412 623 1212.
E-mail address: beumerjh@upmc.edu (J.H. Beumer).

1570-0232/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2005.11.048

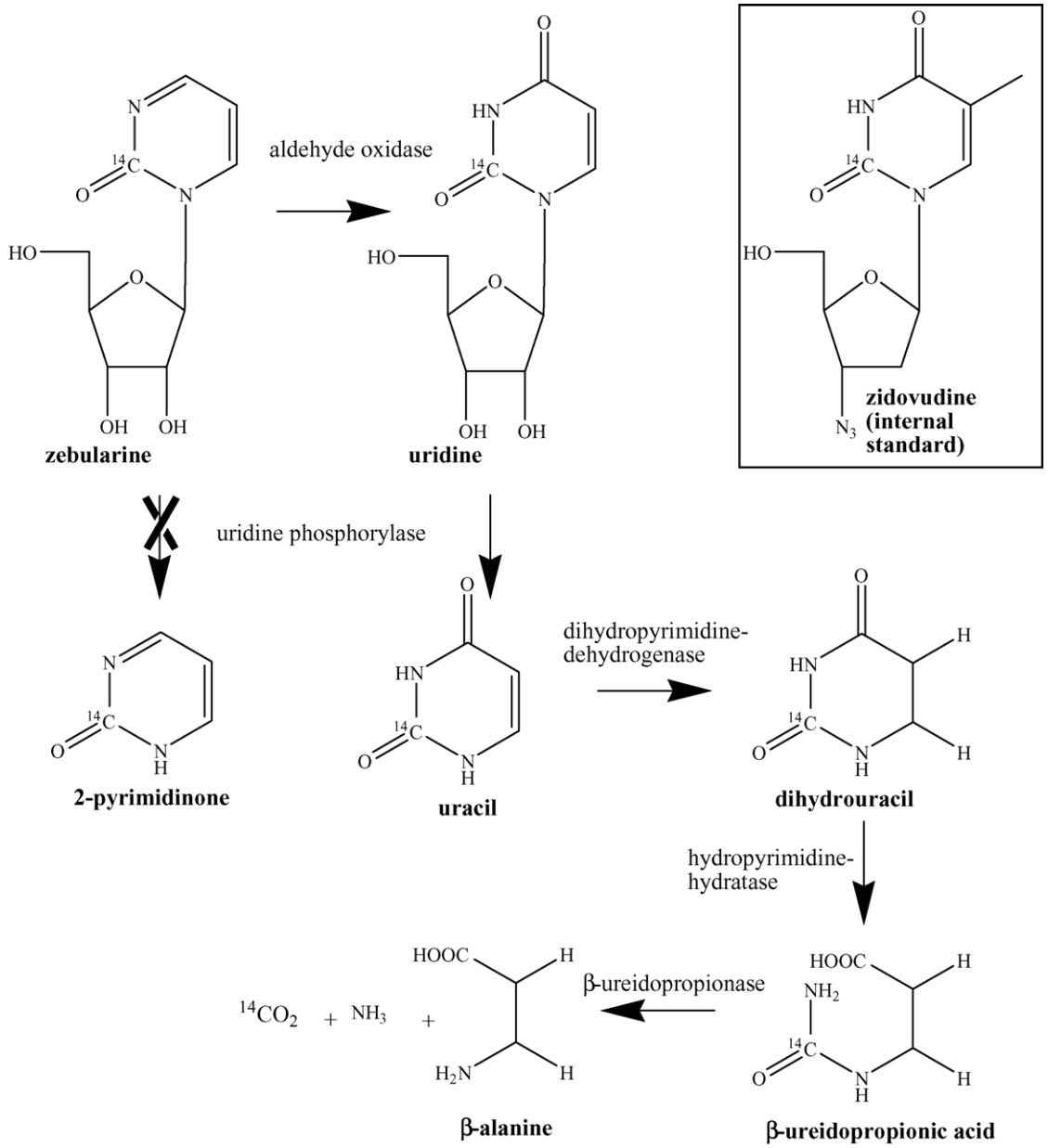

Fig. 1. Chemical structures of zebularine; its main murine metabolites uridine, uracil and dihydrouracil in the proposed metabolic pathway of zebularine; and zidovudine, which was used as internal standard. The "×" indicates the failure to detect 2-pyrimidinone, the potential product of zebularine after loss of the ribose moiety. Eventually, the ^{14}C-label is metabolically released as $^{14}CO_2$.

bon dioxide, ammonia, and β-alanine [9]. Because zebularine is most likely metabolized to endogenous compounds, the use of radiolabeled parent compound is required to allow detection and quantitation of zebularine-derived metabolites. We have used 2-[^{14}C]-zebularine to detect zebularine-derived uridine, uracil, and dihydrouracil in the presence of their endogenous counterparts.

In preparation for a mass balance study of 2-[^{14}C]-zebularine in mice, we have developed an analytical method that allows the simultaneous quantitation of zebularine and its potential metabolites uridine, 2-pyrimidinone, uracil, and dihydrouracil. To this end, we have utilized an HPLC system equipped with tandem UV and radioactivity detection suited to analyze small sample volumes from murine studies.

2. Experimental

2.1. Chemicals and reagents

2-[^{14}C]-Zebularine (purity 99%, 18.3 mCi/mmol) and zebularine were provided by the Developmental Therapeutics Program, National Cancer Institute (Rockville, MD, USA). 2-[^{14}C]-Uridine (purity 99.9%, 52 mCi/mmol), 2-[^{14}C]-uracil (purity 99.5%, 52 mCi/mmol), 2-[^{14}C]-dihydrouracil (purity 98.2%, 53 mCi/mmol), and 2-[^{14}C]-zidovudine (purity 99.8%, 53 mCi/mmol) were obtained as aqueous solutions from Moravek Biochemicals (Brea, CA, USA). Uridine, uracil, dihydrouracil, 2-pyrimidinone, and formic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Zidovudine was a gift

from Burroughs Wellcome (Research Triangle Park, NC, USA). Anhydrous sodium sulfate and ammonium formate were purchased from Fluka (Buchs, Germany). Isopropanol and acetonitrile were obtained from Fisher Chemicals (Fair Lawn, NJ, USA). All chemicals were of analytical grade. Water was purified using a Q-gard® 1 Gradient Milli-Q system (18.2 MΩ cm, Millipore, Billerica, MA, USA). Control murine plasma for the calibration standards was obtained from Lampire Biological Laboratories (Pipersville, PA, USA). Murine plasma for preparation of quality control samples was obtained from CD_2F_1 mice (Taconic, Germantown, NY, USA).

2.2. HPLC

The HPLC system consisted of a Beckman Coulter System Gold 126 solvent module and a System Gold 508 autosampler (Mississauga, Ontario, Canada). Eluent A consisted of an isopropanol:1 M ammonium formate, pH 3.0:acetonitrile (12:0.24:88, v/v/v) mixture. Eluent B consisted of an isopropanol:1 M ammonium formate, pH 3.0:acetonitrile (49:1:50, v/v/v) mixture. After degassing by sonication, these mobile phases were pumped through a Zorbax NH_2 column (5 μm, 250 mm × 4.6 mm I.D., Agilent Technologies, Newcastle, DE, USA) protected by an NH_2 guard column (12.5 mm × 4.6 mm I.D.) at ambient temperature (25 °C) and at a flow rate of 0.6 mL/min. From 0 to 3.0 min, 20% eluent B was pumped through the column. From 3.0 to 8.0 min, a linear gradient to 100% eluent B was applied, followed by 100% eluent B until 26.0 min. The column was reconditioned for the next injection with 20% eluent B from 26.0 to 30.0 min.

2.3. Serial UV and radioactivity detection

The column effluent flowed through a Beckman Coulter System Gold 166 detector, which monitored absorption at 280 nm, and thereafter was mixed in a 1:3 ratio with TrueCount liquid scintillant (IN/US Systems, Tampa, FL, USA) followed by β-decay monitoring by an IN/US Systems β-RAM model-3 radio-HPLC detector, with a 500 μL detector cell. UV-data were captured using Beckman Coulter 32 Karat software version 3.1, while the radioactivity detector data were analyzed using IN/US LabLogic Laura Lite software version 3.3.10.49. The analyte-to-internal standard ratio was calculated by dividing the analyte peak area by the respective internal standard peak area.

2.4. Preparation of calibration standards and quality control samples

The dosing solution to be used in the pharmacokinetic and mass balance study of 2-[^{14}C]-zebularine contained 10 mg/mL zebularine and 150 μCi of 2-[^{14}C]-zebularine/mL. This corresponded to a specific activity of 15 μCi/mg zebularine (3.4 μCi/mmol). Aqueous stock solutions were prepared at 3.4 μCi/mmol for zebularine (10 mg/mL), uridine (4.0 mg/mL), uracil (1.0 mg/mL), dihydrouracil (0.40 mg/mL), and zidovudine (0.14 mg/mL) by mixing adequate amounts of cold and radioactive analytes. These solutions were checked for UV and radio-purity and mixed to obtain a calibration stock solution, which also served as the highest calibration working solution. This solution was serially diluted with water to obtain all the lower calibration working solutions. Aliquots of 20 μL were added to 200 μL of murine plasma to produce the following concentrations: 5.0, 10.0, 15.0, 20.0, 50.0, 75.0, and 100 μg/mL zebularine; 2.5, 5.0, 7.5, 10.0, 25.0, 37.5, and 50.0 μg/mL uridine; 1.0, 1.5, 2.0, 5.0, 7.5, and 10.0 μg/mL uracil; and 0.50, 0.75, 1.0, 2.5, 3.75, and 5.0 μg/mL dihydrouracil. The calibration stock solution was stored at −20 °C.

Quality control (QC) aqueous stock solutions for the individual compounds were prepared independently and mixed to obtain QC stock solutions. Of these stock solutions, 20 μL were added to 200 μL of murine plasma to produce the following concentrations: QC low (QCL) 15.0, 7.5, 2.0, and 1.0 μg/mL zebularine, uridine, uracil, and dihydrouracil, respectively; QC mid (QCM) 35.0, 20.0, 4.0, and 2.0 μg/mL zebularine, uridine, uracil, and dihydrouracil, respectively; QC high (QCH) 80.0, 40.0, 8.0, and 4.0 μg/mL zebularine, uridine, uracil, and dihydrouracil, respectively. The QC stock solutions were stored at −20 °C.

2.5. Sample preparation

To calibration and QC samples (200 μL of plasma with 20 μL of respective stock solution), 25 μL of 2-[^{14}C]-zidovudine internal standard (IS) (0.14 mg/mL) solution were added. After vortexing (10 s), 1 mL of acetonitrile was added to precipitate proteins, the sample was vortexed (10 s) and centrifuged (3 min, 12,000 × *g*, room temperature). Following protein precipitation, approximately 1 g of anhydrous sodium sulfate was added to each sample. The sample was vortexed (10 s) and centrifuged (3 min, 12,000 × *g*, room temperature). The resulting supernatant was decanted into glass tubes and evaporated to dryness under a gentle stream of nitrogen at 40 °C. Sample preparation was carried out on ice until evaporation to dryness. The dried residue was reconstituted in 200 μL of isopropanol: acetonitrile (20:80, v/v) and sonicated for 10 min. The clear supernatant was transferred to an autosampler vial, and 100 μL were injected onto the HPLC system.

2.6. Animals

Specific-pathogen-free, adult CD_2F_1 male mice were purchased from Taconic. Mice were allowed to acclimate to the University of Pittsburgh Cancer Institute Animal Facility for 1 week before being used. To minimize infection, mice were maintained in micro-isolator cages in a separate room and handled in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and on a protocol approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Ventilation and airflow were set to 12 changes per hour. Room temperatures were regulated at 22 ± 1 °C, and the rooms were kept on automatic 12-h light/dark cycles. Mice received Prolab ISOPRO RMH 3000 Irradiated Lab Diet (PMI Nutrition International, St. Louis, MO, USA) and water ad libitum, except on the evening before dosing, when all

food was removed. Mice were 6–8 weeks old at time of dosing. Sentinel animals were maintained in the rooms housing mice, and assayed at 3-month intervals for specific murine pathogens by mouse antibody profile testing (Charles River, Boston, MA, USA). Sentinel animals remained free of specific pathogens, indicating that the study mice were pathogen-free.

2.7. Validation procedures

2.7.1. Calibration curve and lower limit of quantitation (LLQ)

The analytes under investigation were injected at decreasing concentrations to determine the minimal concentration with a signal-to-noise ratio of at least 5:1. Calibration standards (7 for zebularine and uridine, 6 for uracil and dihydrouracil) and blanks were prepared and analyzed in quintuplicate to establish the calibration range with acceptable accuracy and precision. Calibration curves (peak area ratio of the analyte to the internal standard versus the nominal concentration) were fitted by least-squares linear regression with $1/y^2$ (y = response) as the weighting factor. The deviations of these back-calculated concentrations from the nominal concentrations, expressed as percentage of the nominal concentration, reflect the assay performance over the concentration range.

2.7.2. Accuracy and precision

We prepared samples at the LLQ level and QC levels (from stock solutions prepared independent of the calibration stock solutions). The accuracies and precisions for the analytes quantitated by this method were determined by analyzing these samples in a minimum of five replicates in three analytical runs together with an independently prepared, duplicate calibration curve. The accuracy was calculated at each test concentration as: (mean measured concentration/nominal concentration) × 100%. The assay precision was obtained for each test concentration using the coefficient of variation of the measured concentration (all 15 determinations used for inter-assay precision; mean of the three quintuplicate sets used for the intra-assay precision).

2.7.3. Selectivity and specificity

To investigate whether endogenous matrix constituents interfered with the assay, six individual batches of control, drug-free murine plasma were processed and analyzed according to the described procedures. Responses of compounds at the LLQ level were compared with the response in the blank samples.

2.7.4. Recovery

We determined the recoveries of zebularine, uridine, uracil, dihydrouracil and zidovudine from plasma by comparing the response of the detector (expressed as DPM) with the amount of radioactivity initially added to quintuplicate samples at the three QC concentrations.

2.7.5. Stability

We investigated the stability of dihydrouracil, uracil, uridine, and zebularine in the QCH stock solutions after storage at −20 °C for 6 weeks with intermittent thawing and freezing (for use in the assay). The response was compared to a freshly prepared QCH stock solution, and stability was expressed as the percentage recovery of the stored solution relative to the fresh solution. In addition, the stability of dihydrouracil, uracil, uridine, and zebularine in the calibration stock solution at room temperature for 24 h was determined in duplicate. All stability testing in plasma was performed in triplicate at three concentration levels (QCL, QCM, and QCH). We determined the stability of dihydrouracil, uracil, uridine, and zebularine in plasma at −80 °C by assaying samples at $t = 0$ and 6 weeks. The effect of three freeze/thaw cycles on the analyte concentrations in the biological matrices was evaluated by assaying samples after freezing and thawing on 3 separate days and comparing them to freshly prepared samples. The stability of the analytes in the biological matrices during sample preparation (i.e., in the plasma matrix) was evaluated by assaying samples at $t = 0$ and after 2 h on ice. To evaluate the stability of the reconstituted compounds in the autosampler, we compared the response of the calibration set run at the start of every assay with the one run at the end of the respective assay (typically more than 24 h apart). Results of the second runs were expressed as percentage of their respective values in the first runs.

3. Results and discussion

3.1. Method development

The development of an analytical method for zebularine presented a number of challenges that must be addressed in analytical methods development for most anti-metabolites and their metabolic products. The difference in polarity between the ribonucleosides (zebularine and uridine) and the bases (2-pyrimidinone, uracil, and dihydrouracil) required long run-times or the use of a gradient mobile phase. In addition, separation of sets of structurally similar compounds, such as zebularine and uridine or 2-pyrimidinone, uracil and dihydrouracil, required an analytical system with high resolution.

3.1.1. Reverse phase HPLC

Initially, we used two Zorbax SB300 C8 columns (5 μm, 250 mm × 4.6 mm I.D., Agilent Technologies) in series. The mobile phase consisted of acetic acid-sodium acetate buffer (10 mM, pH 5.0) in water and was pumped through the columns at a flow rate of 0.6 mL/min [8]. For detection, UV absorbance was monitored at 280 nm. Different molarities of the buffer (0.01, 0.1, 0.3, and 1.0 M) and various acidities (pH 3.0, 4.0, and 5.0) were tested, resulting in a seemingly suitable system (0.3 M acetic acid–sodium acetate buffer, pH 5.0) with near-base-line separation of 2-pyrimidinone (11.7 min), uracil (13.6 min), uridine (16.8 min), zebularine (17.8 min), and zidovudine (22.1 min). Moreover, reconstitution of the dried supernatant of an acetonitrile deproteination of plasma (1 mL of acetonitrile to 200 μL of plasma) in mobile phase resulted in recoveries of 85–100% for all analytes. However, upon use of this chromatographic system in series with the on-line radioactivity detector, the addition of scintillant to the mobile phase

precipitated the acetate buffer. After comparing the compatibilities of various buffers with the liquid scintillant, we chose ammonium formate as the buffer component. Again, various molarities (including molarity-gradients) and pH's were tested, none of which effected sufficient separation of the analytes.

3.1.2. Hydrophilic interaction chromatography

Because conventional reverse phase HPLC did not result in adequate separation, we tried an amino (NH_2) column, which separates compounds based on hydrophilic interaction liquid chromatography (HILIC) [10]. This type of column is occasionally used to quantitate impurities in pharmaceutical compounds, but its use in the analysis of biological samples is still limited. HILIC employs stationary phases (bare silica or propylamino chains) with a more hydrophilic character than those used in reverse phase HPLC. HILIC is similar to normal phase chromatography in that polar compounds are retained longer than non-polar compounds, and the polar mobile phase component (usually water or methanol) is the strong solvent that decreases analyte retention. Depending on the polarity of the mobile phase, the column is operated in the normal or reverse phase, and, depending on the pH, in the anion-exchange mode. Selectivity can be modified by solvent selection, pH, buffer ions and ionic strength [11]. The mixed-mode of retention provides additional options for modulation of selectivity. However, it also causes unpredictable shifts of peaks upon modifying the mobile phase. In reverse phase chromatography, increasing the fraction of organic solvent generally decreases retention of all analytes. HILIC involves more than a single mechanism of retention (partitioning and ion exchange). Therefore, changing the fraction of organic solvent can affect the retention of individual compounds to different degrees, resulting in concurrent increased retention for one compound and decreased retention for another compound. This was observed during the present method development (data not shown). HILIC seemed a potentially viable approach for chromatographic separation of zebularine and its metabolites as it had been reported effective in the chromatography of uracil, a proposed metabolite of zebularine, and a compound that is often used in reverse phase chromatography to indicate the void volume [10].

3.1.3. Method development based on HILIC

An initial scout gradient from 95% (v/v) acetonitrile to 60% (v/v) acetonitrile in 10 mM aqueous ammonium formate, pH 5.0, resulted in a quite different elution order (first zidovudine, followed by uracil, uridine, 2-pyrimidinone, and finally zebularine) than that observed using the aforementioned reverse phase system. The latter three compounds eluted in a 3-min time frame and needed to be resolved better. However, while optimizing the gradient system, it was noted that the separation of these compounds gradually worsened, and initial results could not be reproduced. Because HILIC columns need a large number of column volumes to equilibrate properly, we considered the solvents to which the column had been exposed. We realized that the column had been delivered filled with hexane, which was eluted using isopropanol prior to use (according to the manufacturers' instructions). Perfusing the column with a few milliliters of isopropanol, followed by removing the isopropanol with the acetonitrile: ammonium formate mobile phase, restored separation of the analytes on our system. Therefore, we tested various combinations of isopropanol: water as the more polar components of the mobile phase. Although the addition of isopropanol improved separation, the peaks of uridine and zebularine were non-symmetrical and showed extensive tailing. Decreasing the pH from 5.0 to 3.0 improved peak shape and finally resulted in the mobile phases used. This clearly demonstrates the dependence of retention on column history, an observation that was initially described for HILIC employing unbuffered aqueous mobile phases [10]. However, our data indicate that it also applies to organic solvents previously pumped through the column. This phenomenon obviously deserves special attention with respect to long-term chromatographic reproducibility during the development of assays based on HILIC.

Reconstitution of dried residue in the mobile phase used at the start of the run (200 μL of 20% isopropanol in acetonitrile) resulted in unsatisfactory recovery. Addition of 10% water improved reconstitution, but caused distortion of the peak shape of uracil. Eventually we chose to remove water by adding approximately 1 g of anhydrous sodium sulfate to the plasma-acetonitrile supernatant. Presumably, because fewer plasma components dissolved in this dehydrated supernatant, the dried residue was more easily dissolved in the reconstitution solvent. The relatively high injection volume of 100 μL was chosen to improve sensitivity and was shown to yield good chromatographic behavior.

Initially, sample preparation was performed at room temperature. However, uridine was unstable in plasma over 4 h at room temperature (64.3, 66.9, and 89.2% remaining at the QCL, QCM, and QCH levels, respectively). The decreased stability of uridine in comparison to the other compounds under investigation may explain why the assay performance of uridine was less than adequate (data not shown). Consequently, sample preparation was performed on ice.

3.2. Validation of the assay

3.2.1. Calibration curve and LLQ

The calibration curve describes the concentration-response relationship adequately if the observed deviation and precision are $\leq$20% for the LLQ and $\leq$15% for all other calibration concentrations. At least four of six calibration points should meet the above criteria [12].

A weighting factor of $1/y^2$ was used in constructing the calibration curves, resulting in a better fit for the lower concentrations. The selected assay ranges (5–100 μg/mL for zebularine, 2.5–50 μg/mL for uridine, 1–10 μg/mL for uracil and 0.5–5.0 μg/mL for dihydrouracil) complied with the FDA criteria for the LLQ and the calibration curve. Representative calibration curves and corresponding correlation and regression coefficients are shown in Fig. 2.

3.2.2. Accuracy and precision

FDA guidelines specify that the accuracies for all tested concentrations should be within ±15%, and the precisions should

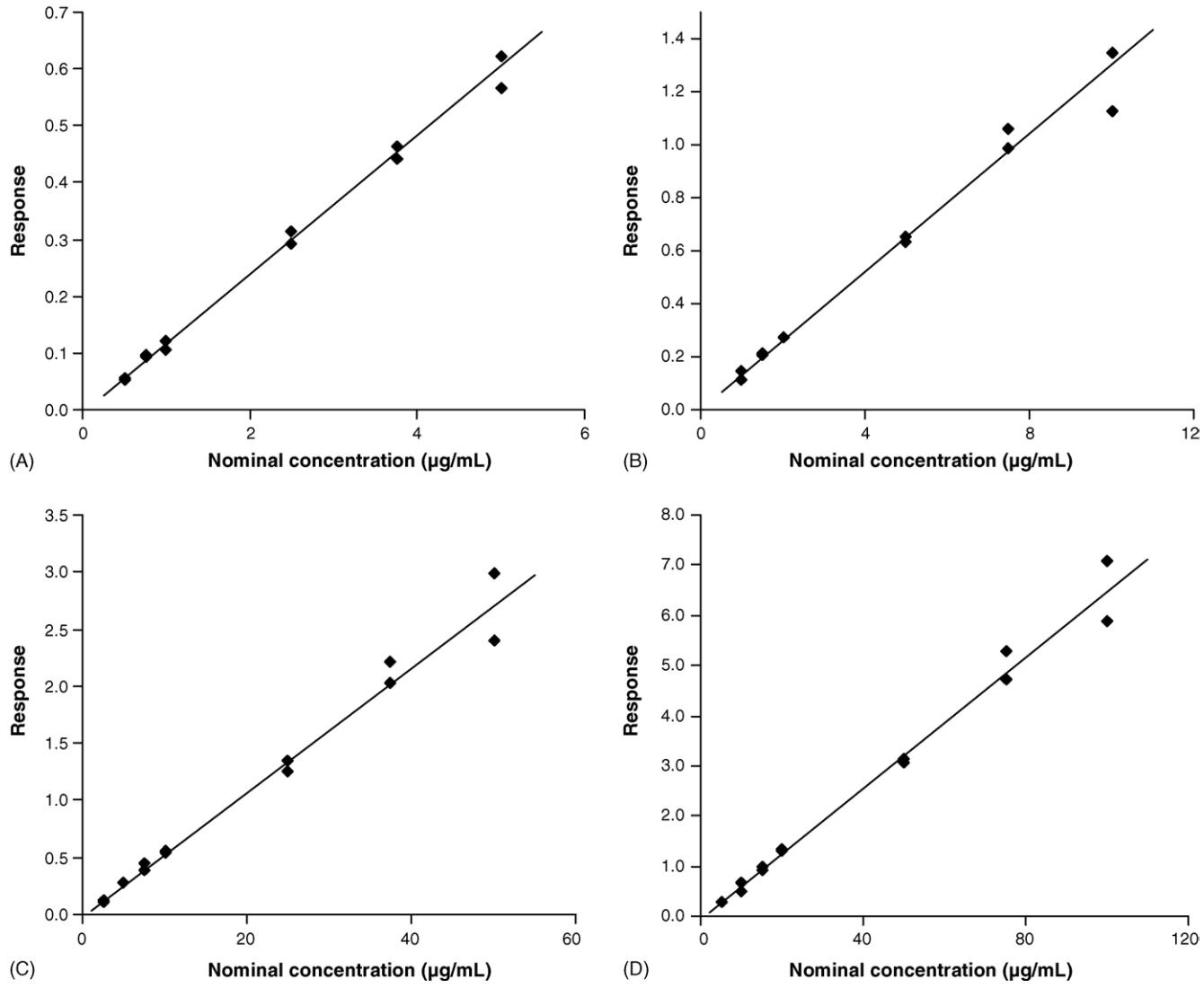

Fig. 2. Representative calibration curves of dihydrouracil ((A) response = 0.122 × conc − 0.005; R^2 = 0.991), uracil ((B) response = 0.130 × conc + 0.003; R^2 = 0.982), uridine ((C) response = 0.054 × conc − 0.022; R^2 = 0.977), and zebularine ((D) response = 0.065 × conc − 0.052; R^2 = 0.989).

not exceed 15% except for the LLQ, in which case these parameters should not exceed 20% [12].

The accuracies and intra- and inter-assay precisions for the tested concentrations (LLQ, QCL, QCM, QCH) were all within the pre-set acceptance criteria (Table 1), except for uridine which displayed an inter-assay precision of 15.7% at the QCM level. Although not within the FDA-specified limits, this deviation was deemed negligible.

3.2.3. Selectivity and specificity

According to FDA guidelines, the signal at the LLQ must be at least five times the signal of any co-eluting peaks [12,13].

Radiochromatograms of six individual control plasma samples contained no co-eluting peaks >5% of the areas at the LLQ. Representative chromatograms of control plasma and plasma with analytes at the LLQ level are displayed in Fig. 3. Typical retention times and capacity factors are listed in Table 2. In subsequent analyses, we did not observe any interfering or co-eluting peaks.

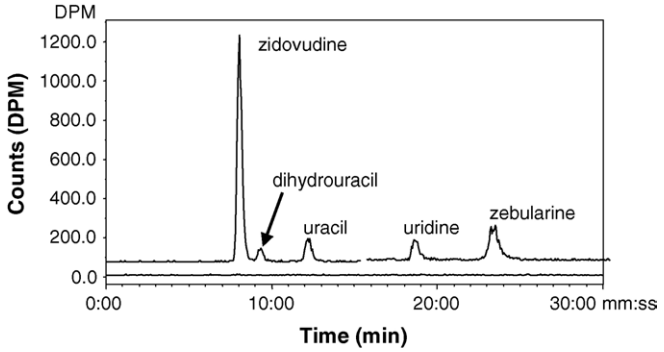

Fig. 3. Representative radio-chromatograms of control murine plasma (lower tracing), and zidovudine (IS, 8.0 min), dihydrouracil (9.3 min), uracil (12.3 min), uridine (18.8 min), and zebularine (23.5 min) added to control plasma at the LLQ level. Signals from the sample at the LLQ have an offset of 80 DPM.

Table 1
Assay performance data for the quantitation of dihydrouracil, uracil, uridine, and zebularine in murine plasma at the LLQ, QCL, QCM, and QCH levels

Concentration (μg/mL)	Accuracy (%)	Intra-assay precision (%)	Inter-assay precision (%)
Dihydrouracil			
0.5	98.4	9.7	10.5
1.0	102.7	7.7	8.3
2.0	100.7	5.0	5.6
4.0	96.9	3.8	5.0
Uracil			
1.0	98.2	6.0	6.2
2.0	103.4	7.8	8.4
4.0	100.5	8.6	9.8
8.0	98.6	8.3	8.9
Uridine			
2.5	106.9	5.4	17.0
7.5	98.9	12.8	13.8
20.0	100.2	13.2	15.7[a]
40.0	92.2	10.7	11.0
Zebularine			
5.0	106.8	6.0	13.2
15.0	102.4	9.5	11.2
35.0	101.8	11.1	12.4
80.0	100.3	9.4	9.5

$N = 15$.
[a] Value falls outside of FDA limit (15%).

3.2.4. Recovery

FDA-guidelines require that recovery is consistent and precise. A recovery of ≥70% with a variation of 15% is generally accepted [12,13].

The recoveries of zebularine, uridine, uracil, and dihydrouracil at the three QC concentrations and zidovudine ranged from 9.3 (uridine) to 17.4%, with a relative standard deviation of 3.4–17.9% (uridine). The recovery of parent compound zebularine was 13.3–16.6% with a relative standard deviation of 4.4–9.9%. Results are shown in Table 3. The low recovery is presumably caused by the use of anhydrous sodium sulfate during the sample preparation and the use of a non-aqueous reconstitution solvent. The latter is one of the drawbacks of HILIC, which employs non-polar mobile phases to reconstitute polar compounds. As the assay generally fulfilled the FDA-specified criteria for precision and accuracy, this low recovery was deemed acceptable.

Table 2
Typical retention times and capacity factors for dihydrouracil, uracil, uridine, and zebularine, internal standard zidovudine, and 2-pyrimidinone employing the described assay

Analyte	Retention time (min)	Capacity factor
Dihydrouracil	9.4	0.97
Uracil	12.5	1.6
Uridine	18.9	3.0
Zebularine	24.2	4.1
Zidovudine	8.0	0.68
2-Pyrimidinone	20.7	3.3

Table 3
Recovery of dihydrouracil, uracil, uridine, zebularine, and zidovudine from murine plasma

Concentration (μg/mL)	Recovery (%)	R.S.D. (%)
Dihydrouracil		
1.0	17.4	5.4
2.0	14.0	11.2
4.0	14.4	12.9
Uracil		
2.0	17.3	3.4
4.0	13.6	9.6
8.0	14.7	13.2
Uridine		
7.5	14.2	7.7
20.0	9.3	17.9
40.0	10.7	9.0
Zebularine		
15.0	16.6	4.5
35.0	13.3	10.0
80.0	14.7	7.7
Zidovudine		
–	16.1	10.5

$N = 5$.

3.2.5. Stability

Stability in biological samples is acceptable when ≥85% of the analyte is recovered.

The stabilities of dihydrouracil, uracil, uridine, and zebularine in stock solutions at −20 °C, for 6 weeks with intermittent thawing for use, were 98.5, 103.0, 105.7, and 99.1%, respectively ($N = 3$). At room temperature for 24 h, the stabilities of these compounds in stock solution were 105.1, 102.7, 107.9, and 107.0%, respectively ($N = 3$). The stabilities of dihydrouracil, uracil, uridine, and zebularine in plasma during freeze-thaw cycling and for 6 weeks at −80 °C are shown in Tables 4 and 5,

Table 4
Stability of dihydrouracil, uracil, uridine, and zebularine in plasma during three consecutive freeze–thaw cycles (−80 °C to room temperature) at the QCL, QCM, and QCH levels

Concentration (μg/mL)	Stability (%)	R.S.D. (%)
Dihydrouracil		
1.0	96.2	11.7
2.0	104.7	4.7
4.0	100.0	7.7
Uracil		
2.0	103.5	5.7
4.0	111.5	5.7
8.0	100.5	12.2
Uridine		
7.5	103.2	12.4
20.0	109.3	9.7
40.0	94.8	13.6
Zebularine		
15.0	103.8	8.2
35.0	112.9	5.0
80.0	97.3	11.2

$N = 3$.

Table 5
Stability of dihydrouracil, uracil, uridine, and zebularine in plasma for 6 weeks at −80 °C at the QCL, QCM, and QCH levels

Concentration (μg/mL)	Stability (%)	R.S.D. (%)
Dihydrouracil		
1.0	97.9	11.2
2.0	98.1	7.9
4.0	99.9	7.6
Uracil		
2.0	97.7	4.8
4.0	97.6	5.1
8.0	94.5	10.8
Uridine		
7.5	86.0	12.7
20.0	94.4	14.7
40.0	91.7	11.3
Zebularine		
15.0	88.8	9.7
35.0	95.1	12.1
80.0	105.3	9.1

$N = 3$.

respectively. Dihydrouracil, uracil and zebularine proved to be sufficiently stable in plasma on ice (Table 6). The recoveries of dihydrouracil, uracil, uridine, and zebularine reconstituted and kept in the autosampler for more than 24 h were 104.8, 107.8, 104.7, and 107.7%, respectively.

3.3. Application of the assay to biological samples

To show the applicability of the method, we dosed two specific-pathogen-free, adult, male CD_2F_1 mice of approximately 20 g body weight with 100 mg/kg zebularine i.v., corresponding to 15 μCi/mg zebularine (approximately 30 μCi per mouse). Individual mice were euthanized by CO_2 inhalation at 5 min or 2 h after dosing. Heparinized blood was collected by

Table 6
Stability of dihydrouracil, uracil, uridine, and zebularine in plasma for 2 h on ice at the QCL, QCM, and QCH levels

Concentration (μg/mL)	Stability (%)	R.S.D. (%)
Dihydrouracil		
1.0	105.8	11.5
2.0	102.7	12.1
4.0	109.4	19.0
Uracil		
2.0	106.5	6.4
4.0	100.4	9.4
8.0	108.3	8.6
Uridine		
7.5	113.5	14.4
20.0	117.7	22.4
40.0	105.9	18.6
Zebularine		
15.0	113.7	10.4
35.0	108.1	15.3
80.0	102.9	17.6

$N = 3$.

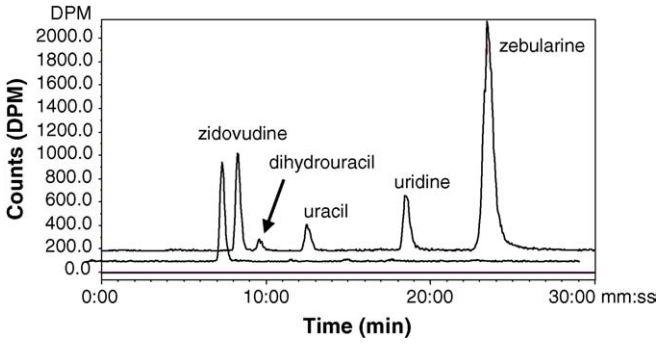

Fig. 4. Radiochromatograms of plasma samples obtained 5 min (200 DPM offset) and 120 min (100 DPM and −1 min offset) after dosing mice with 100 mg 2-[^{14}C]-zebularine/kg.

cardiac puncture and centrifuged at 13,000 × *g* at room temperature for 5 min to obtain plasma, which was stored at −80 °C until analysis. For analysis, 20 μL of water and 25 μL of 2-[^{14}C]-zidovudine IS solution were added to 200 μL of plasma sample, followed by the sample preparation as described above.

The 5-min sample contained 69.7 μg/mL zebularine, 13.4 μg/mL uridine, 2.9 μg/mL uracil, and 1.1 μg/mL dihydrouracil (Fig. 4). The other potential metabolite, 2-pyrimidinone, was not observed, suggesting zebularine is converted to uridine by aldehyde oxidase prior to any subsequent metabolic degradation (Fig. 1). Therefore, 2-pyrimidinone was not included as an analyte in the method validation. None of the analytes of interest were observed in the 2-h plasma sample. Thus, overall metabolic degradation of zebularine and its metabolites is rapid.

4. Conclusion

Our objective was to develop an analytical method for the identification and quantitation of zebularine and its metabolites. We accomplished this using hydrophilic interaction chromatography (HILIC) to separate the analytes of interest. HILIC seems suited to analyze anti-metabolites that are structurally related to zebularine, and this analytical technique may also be useful for quantitation of drugs like gemcitabine [14], 5-fluorouracil and its pro-drugs capecitabine and 5-fluorodeoxyuridine, and their respective metabolites.

The method presented here allows the simultaneous quantitation of zebularine and its metabolites uridine, uracil and dihydrouracil in plasma and meets the FDA guidelines for all analytes except uridine [12]. Because only one (QCM) of four concentrations tested barely failed the criterium for precision (15.7% as apposed to the limit of 15%), the assay parameters for uridine were still deemed adequate for employing this method to support pharmacokinetic studies. 2-Pyrimidinone, a potential metabolite of zebularine, was not detected. Using the present assay, we have shown that zebularine administered to mice is metabolized to uridine, entering the pyrimidine nucleoside catabolic pathway [9]. Overall elimination of zebularine and its metabolites in mice appears to be rapid. Naturally these findings need confirmation, and an appropriately designed pharmacokinetic study is ongoing.

Future studies will employ this analytical assay and the knowledge about the metabolic pathway of zebularine to elucidate further the disposition of zebularine and metabolites in murine plasma. Finally, the analytical method presented in this paper may be adapted to support clinical studies with zebularine.

Acknowledgements

Supported by contract N01-CM-52202 from the National Cancer Institute. We thank the UPCI Hematology/Oncology Writing Group for constructive suggestions regarding the manuscript. We thank Diane Mazzei and her colleagues at the University of Pittsburgh Animal Facility for their expert assistance.

References

[1] J. Laliberte, V.E. Marquez, R.L. Momparler, Cancer Chemother. Pharmacol. 30 (1992) 7.
[2] L. Zhou, X. Cheng, B.A. Connolly, M.J. Dickman, P.J. Hurd, D.P. Hornby, J. Mol. Biol. 321 (2002) 591.
[3] P.A. Jones, S.B. Baylin, Nat. Rev. Genet. 3 (2002) 415.
[4] C.B. Yoo, J.C. Cheng, P.A. Jones, Biochem. Soc. Transact. 32 (2004) 910.
[5] M. Lemaire, L.F. Momparler, M.L. Bernstein, V.E. Marquez, R.L. Momparler, Anticancer Drugs 16 (2005) 301.
[6] J.C. Cheng, C.B. Matsen, F.A. Gonzales, W. Ye, S. Greer, V.E. Marquez, P.A. Jones, E.U. Selker, J. Natl. Cancer Inst. 95 (2003) 399.
[7] J.L. Holleran, R.A. Parise, E. Joseph, J.L. Eiseman, J.M. Covey, E.R. Glaze, A.V. Lyubimov, Y.F. Chen, D.Z. D'Argenio, M.J. Egorin, Clin. Cancer Res. 11 (2005) 3862.
[8] R.W. Klecker, R.L. Cysyk, J.M. Collins, Proc. Am. Assoc. Cancer Res. 46 (2005).
[9] D. Voet, J.G. Voet (Eds.), Biochemistry, John Wiley & Sons Inc., New York, 1995, p. 795, Ch. 26.
[10] B.A. Olsen, J. Chromatogr. A 913 (2001) 113.
[11] Agilent Zorbax NH2 datasheet (2003). http://www.chem.agilent.com/temp/radE41B2/00044450.pdf.
[12] U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), and Center for Veterinary Medicine (CVM), Guidance for Industry – Bioanalytical Method Validation (2001). http://www.fda.gov/cder/guidance/4252fnl.pdf.
[13] H. Rosing, W.Y. Man, E. Doyle, A. Bult, J.H. Beijnen, J. Liquid Chromatogr. Rel. Technol. 23 (2000) 329.
[14] B. Yilmaz, Y. Kadioglu, Y. Aksoy, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 791 (2003) 103.