Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 844 (2006) 15–22
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Validation of an HPLC method for analysis of DB-67 and its water soluble prodrug in mouse plasma

Jamie Horn, Sherri L. Jordan, Lin Song, Michael J. Roberts, Bradley D. Anderson, Markos Leggas *

Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, KY 40536, USA

Received 3 January 2006; accepted 22 June 2006
Available online 21 July 2006

Abstract

A method for the quantitation of DB-67 ((20*S*)-10-hydroxy-7-*tert*-butyldimethylsilylcamptothecin) lactone and carboxylate in mouse plasma has been developed, validated, and applied in pharmacokinetic studies. The analytes were separated by reversed-phase chromatography with fluorescence detection. Validation demonstrated the selectivity and specificity for the carboxylate and lactone, with linearity between 1–300 ng/mL and 2.5–300 ng/mL for the carboxylate and lactone, respectively (accuracy 90–110% of theory and coefficient of variation ≤5.7%). Carboxylate to lactone conversion was <4% using this method. The assay was found to be suitable for the analysis of DB-67 lactone and carboxylate in pharmacokinetic studies following intravenous administration of DB-67 or its δ-aminobutyric acid ester derivative.

Keywords: DB-67; Camptothecin analogs; Method validation

1. Introduction

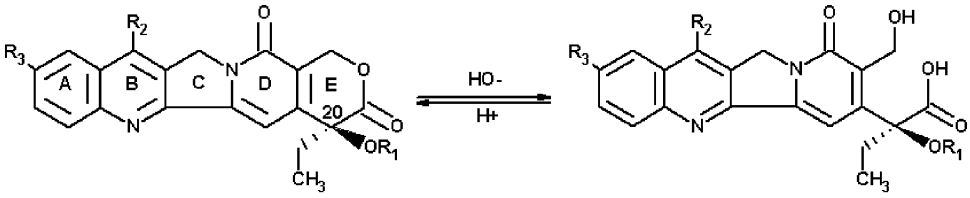
Camptothecin (CPT) and its α-hydroxy-δ-lactone containing analogues are S-phase specific cancer chemotherapeutic agents, which bind and stabilize the DNA/DNA topoisomerase I (TOPI) complex formed during replication [1–4]. The resulting accumulation of single and subsequent double strand breaks is lethal to actively replicating cells [5]. The therapeutic efficacy of these compounds is dependent upon the continuous exposure of total tumor cell population to membrane soluble "closed-ring," lactone forms of these molecules, which exist in equilibrium with their pharmacologically inactive carboxylate forms (Fig. 1; [6–8]). Thus, the activity of these compounds is dictated by the dynamics of the reversible hydrolysis reaction, which is known to be influenced by pH as well as by binding of the carboxylate to albumin, particularly human serum albumin (HSA) [9].

Structure–activity studies have demonstrated that A- and B-ring modifications to the CPT ring system can influence HSA binding while conserving anti-TOPI activity [10]. Such studies were result of the modest efficacy and unacceptable toxicity displayed by CPT in early clinical trials; traits which were eventually attributed to its poor solubility, limited bioavailability (due to HSA binding) and rapid hydrolysis to the carboxylate form [11]. Consequently, a number of second-generation CPT derivatives were designed containing A- and/or B-ring functional groups aimed at improving aqueous solubility and bioavailability. One primary example is the FDA-approved prodrug irinotecan (CPT-11), which is metabolized by carboxylesterases to form SN-38. The active SN-38 metabolite is a 7-ethyl-10-hydroxy substituted CPT analogue (see Fig. 1), which displays ∼20% lactone levels in human blood as compared to ∼5% for CPT [12].

More recent drug development efforts have investigated the effect of A- and B-ring silylalkyl substitutions on lactone stability [12–14]. One of the most promising candidates to emerge from such studies, namely (20*S*)-10-hydroxy-7-*tert*-butyldimethylsilylcamptothecin (DB-67), contains dual 7-, 10-type substitutions similar to SN-38 (see Fig. 1). It was rationalized that 7-silylalkyl substitution would enhance lipophilicity and protect circulating lactone via reversible partitioning into lipid bilayers. Furthermore, this substitution would disrupt carboxylate affinity for HSA. In fact, DB-67 has been shown to display higher lactone levels in human blood than SN-38 (∼30% versus ∼20%) while still retaining potent cytotoxic activity

* Corresponding author. Tel.: +859 257 2633; fax: +859 257 7564.
E-mail address: mark.leggas@uky.edu (M. Leggas).

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.06.022



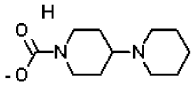
Compound	R1	R2	R3
Camptothecin (CPT)	H	H	H
CPT-11	H	$-CH_2CH_3$	
SN-38	H	$-CH_2CH_3$	-OH
DB-67	H	$-Si(CH_3)_2C(CH_3)_3$	-OH
DB-67-AB	$-CO(CH_2)_3NH_2$	$-Si(CH_3)_2C(CH_3)_3$	-OH

Fig. 1. Molecular structures of the lactone (left) and carboxylate (right) forms of camptothecins. Alphanumeric identification of the camptothecin ring system and the 20-C within the ring system is given. Particular camptothecins are identified according to their R_1, R_2 and R_3 substitution groups.

[12,15]. This drug is now entering into Phase I clinical trials under RAID governance, while translational and basic research for appropriate and efficient delivery forms is still ongoing.

One strategy for prolonged exposure of cancers to lipophilic camptothecin analogues is the administration of ester prodrug forms loaded into liposomes, which can passively accumulate within solid tumor interstial space [16]. Under physiologic conditions, 20-OR ester prodrugs undergo chemical, rather than enzymatic, hydrolysis (for detailed hydrolysis mechanism see [16]). This property makes them good candidates for delivery with liposomal systems as drug release at sites of liposome accumulation is independent of any interpatient variability in enzyme expression. Studies involving the prototype DB-67 prodrug 20-(*S*)-4-aminobutyric acid ester derivative (DB-67-AB; see Fig. 1) demonstrate that it is a likely candidate for this type of passive DB-67 delivery as it can be efficiently loaded into liposomal cores at low pH and, in whole blood, is retained within liposomes for periods up to 40 h [16].

As DB-67 and other third generation camptothecin analogues have progressed to clinical trials and as significance of the lactone–carboxylate equilibrium has become more apparent, methods to simultaneously quantify circulating lactone and carboxylate levels have become increasingly important. Whereas early HPLC methodologies typically measured total camptothecin analogue levels, more recent efforts have focused on determining conditions necessary for independently quantifying the levels of both forms in plasma [17–21]. These methods typically involve reverse-phase chromatography with isocratic elution by mobile phase containing buffer, for ionic strength and pH control, acetonitrile (ACN), for mobile-phase strength adjustment, and ion-pairing reagent for carboxylate retention [17,19,20,22].

The current work details the development of a similar method for the analysis of the novel lipophilic camptothecin analog, DB-67, as well as its hydrophilic prodrug, DB-67-AB. This method was optimized for quantitating DB-67 carboxylate and lactone forms in mouse plasma, validated and applied to pharmacokinetic studies. Partial validation to incorporate DB-67-AB as an analyte has been included to demonstrate the capability of the assay to simultaneously measure hydrophilic camptothecin prodrugs in addition to the parent lipophilic drug.

2. Experimental

2.1. Materials

All reagents and chemicals were of the highest purity available. Ammonium acetate (Mallinckrodt Baker, Phillipsburg, NJ), HPLC grade acetonitrile and HPLC grade methanol (Burdick and Jackson, Muskegon, MI) were purchased from VWR. Tetrabutylammonium dihydrogen phosphate (TBAP; 1.0 M aq. solution) was obtained from Sigma–Aldrich (St. Louis, MO), whereas ≥99.7% dimethylsulfoxide (DMSO) came from Fisher Scientific (Fair Lawn, NJ). Blank mouse plasma used in the preparation of calibrators and quality control solutions was from Abacell Corp. (San Mateo, CA). Consumables were treated with AquaSil™ siliconizing reagent (Pierce, Rockford, IL). Siliconized pipet tips were obtained from Cole-Parmer and amber siliconized microcentrifuge tubes from Crystalgen Inc. (Plainview, NY). Magnesium- and calcium-free 10× Dulbecco's phosphate buffered saline was obtained from Gibco (Invitrogen Corp., Carlsbad, CA), diluted to 1× and brought to pH 7.4.

DB-67 of ≥98% purity was obtained from Novartis Pharmaceuticals Corporation. The compound was stored at 4 °C, and handled as if it were a known chemical carcinogen. DB-67-AB was synthesized at ≥95% purity according to a previously published procedure [16].

2.2. Preparation and handling of stock and working solutions

Stock solutions of DB-67 were prepared in DMSO (1 mg/mL) and small volume aliquots for single-time use were stored at −80 °C until required. Two serial dilutions from the stock, with

either 0.005 N NaOH or 0.005 N HCl, were made to generate 10 and 1 μg/mL solutions of the carboxylate and lactone forms, respectively. Aqueous solutions of DB-67 were prepared in amber containers and stored on ice. The more concentrated 10 μg/mL working solutions of the carboxylate and lactone were prepared and equilibrated for 1 h on ice prior to their use in the preparation of high end calibrators and further dilution to 1 μg/mL for low end calibrator preparation. These high and low working solutions were subsequently discarded.

2.3. Preparation of calibration standards and quality control samples

During the preparation of calibrators and quality control samples, all aqueous diluents and solutions were kept on ice and all methanolic diluents and solutions were kept on dry ice. Additionally, the calibrators and quality control samples were made and extracted individually. Plasma calibrators containing 1, 5, 10, 20, 50, 100, 200 or 300 ng/mL of DB-67 carboxylate and 2.5, 5, 10, 20, 50, 100, 200 or 300 ng/mL lactone were prepared by the addition of appropriate volumes of the individual working solutions to 920 μL of blank mouse plasma, followed by dilution to 1000 μL with ice cold magnesium and calcium free 1× Dulbecco's phosphate buffered saline (pH 7.4). Samples were vortexed for 10 s, then a 200 μL aliquot was extracted with four equivalents of methanol which had been previously cooled to −80 °C and kept on dry ice. The extraction mixtures were vortexed for 5 s followed by centrifugation at 13,000 × *g* for 2 min (4 °C). The methanol extracts were collected into separate amber tubes and stored on dry ice or at −80 °C until analysis.

High-, mid- and low-level quality control samples contained 250 (high-QC), 150 (mid-QC) or 25 ng/mL (low-QC) each of the DB-67 analytes. These samples were prepared and extracted in a manner similar to that used for preparation of the calibrator extracts. These extracts were used to validate linear regressed and weighed calibration curves and to confirm daily system suitability throughout the analysis of experimental samples.

2.4. Pharmacokinetic experiments in mice

To obtain DB-67 pharmacokinetic profiles, 18 male C57BL/6 mice (Charles Rivers Laboratories), between 32 and 34 days old, were given a tail vein injection of DB-67 (1 mg/kg) solubilized to 0.2 mg/mL in 22.2% (w/v) sulfobutyl ether β-cyclodextrin (SBE-CD (Captisol®)) in 5% aq. dextrose buffered with 2 mM acetic acid/8.37 mM HCl [23]. Animals were sacrificed at 5 min, 15 min, 1 h, 3 h, 6 h and 8 h ($n = 3$). Blood samples, collected by cardiac puncture into heparinized syringes, were immediately centrifuged and the resulting plasma immediately extracted according to the procedure given for the extraction of calibrators. The organic extracts were collected into amber tubes and placed on dry ice or at −80 °C until analysis. Analysis was done within one week of sample extract preparation.

To obtain DB-67-AB pharmacokinetic profiles, 12 male, 42-day-old CB17/ICR:HSD-SCID mice (Charles Rivers Laboratories) were implanted in their right flanks with 10^6 SJG2 glioblastoma cells [24]. Two weeks later, the xenografted mice were given a tail vein injection of DB-67-AB (1 mg/kg) as a 0.2 mg/mL solution in 10% Diluent 12® in PBS, pH 3.0. Blood collected by cardiac puncture at 5 min, 30 min, 1 h and 6 h ($n = 3$ for each time point) was immediately processed as described for the DB-67 study.

2.5. Instrumentation

Analysis of calibrator, quality control and experimental sample extracts were performed on a Shimadzu HPLC system (Shimadzu Inc., Atlanta, GA) controlled by Class-VP integrating software (version 7.2.1). The system consisted of an in-line degasser (DGU-14A), a LC-10AD VP pump, a refrigerated autoinjector (Shimadzu SIL-10_{AD} VP) with rack temperature at 4 °C, a fluorescence detector (RF-10_{XL}) set at 380 nm excitation/560 nm emission [15], and a mobile-phase recycler.

2.6. Development of chromatographic conditions

The current method was developed on a room temperature, guard-protected reversed-phase C18 analytical column (Waters Nova-Pak C18 4 μm; 3.9 mm × 150 mm) using a mobile-phase mixture of ammonium acetate (NH_4OAc) buffer containing ion-pairing reagent, TBAP, and acetonitrile. The ion-pairing reagent concentration and buffer strength were adjusted to obtain optimum retention time and carboxylate peak shape, respectively. Analytes were eluted isocratically at 1 mL/min using a mobile-phase mixture of 0.15 M NH_4OAc containing 10 mM TBAP (pH 6.5): ACN (65:35, v/v). Unless otherwise indicated sample extracts were diluted with an equivalent volume of 0.15 M NH_4OAc containing 10 mM TBAP (pH 6.5) prior to a 50 μL injection. Under the specified conditions, the carboxylate and lactone eluted at 3.2 and 9.9 min, respectively. Buffer diluted extracts were injected within 3 h of preparation.

2.7. Validation

The method was validated according to the FDA "Guidance for Industry: Bioanalytical Method Validation" document [25]. In general, recommendations were followed except that three, rather than six, selectivity source samples were utilized.

2.8. Recovery and analyte inter-conversion

The recovery of analytes was determined from peak height comparisons of spiked plasma aliquots extracted with methanol (25 and 250 ng/mL levels) to analytes dissolved in methanol (25 and 250 ng/mL levels) both of which were diluted 1:1 with mobile-phase buffer. Duplicate injections of duplicate samples at high and low analyte concentrations were made and the average percent recovery was determined. The inter-conversion of the carboxylate and lactone forms during the preparation and analysis was investigated independently for each analyte in mouse plasma extract, acid/base extract, unextracted acid/base, and in the same samples not diluted with mobile-phase buffer (25 and 250 ng/mL levels, $n = 2$ for each level).

Table 1
Parameters from linear regression analysis of simultaneously generated calibration curves of DB-67 carboxylate (1–300 ng/mL) and lactone (2.5–300 ng/mL) in mouse plasma

	Mean	SD	RSD (%)	*n*
Carboxylate				
Slope	0.0149	0.0029	19.6	3
y-intercept	0.152	0.041	26.3	3
r^2	0.993	0.010	1.01	3
Lactone				
Slope	0.011	0.0013	11.2	3
y-intercept	0.46	0.14	30.3	3
r^2	0.997	0.002	0.20	3

SD: standard deviation. RSD: relative standard deviation.

2.9. Pharmacokinetics

Compartmental pharmacokinetic methods were used to analyze the DB-67 and DB67-AB plasma concentration versus time data. Compartmental modeling was done with the WinNonlin Professional Edition version 4.1 pharmacokinetic modeling program (Pharsight, MountainView, CA, USA). Parameters estimated by the model included the inter-compartmental rate constants for each analyte (k_{12} and k_{21}), the elimination rate constants (k_{10}), and the apparent volume of the central compartment (V_c).

2.10. Statistical analysis

Data and regression analyses were performed with Shimadzu Class VP software. Peak heights were weighted by dividing the squares of the differences of the observed and calculated values by the square of the response (carboxylate) or the response itself (lactone).

3. Results

3.1. Assay development and robustness

The conditions employed for the DB-67 assay were adapted from those used to separate CPT-11 and SN-38 lactone and carboxylate forms [22]. The aqueous buffer:ACN ratio was adjusted to elute the more lipophilic DB-67 carboxylate and lactone within 12 min. In fact, the high sensitivity of analyte retention time to organic solvent content (e.g. as ACN percentage changed from 30 to 37.5%, carboxylate retention time changed from 6.5 to 1.9 min, and lactone retention time from 22.3 to 6.9 min), required great care in mobile-phase preparation to ensure peak height reproducibility throughout the method validation and experimental sample analyses. Additionally, tailing of the carboxylate analyte in mobile phase containing 0.075 M NH_4OAc (pH 6.5) was unaffected by a two-fold increase in ion-pairing reagent, whereas a subsequent change to 0.15 M ammonium acetate (pH 6.5) greatly diminished tailing. The final mobile phase that was validated consisted of a 65:35 mixture of 0.15 M NH_4OAc containing 10 mM TBAP (pH 6.5): ACN.

3.2. Separation and specificity

The carboxylate and lactone were added to three independent samples of blank mouse plasma. These analytes were found to be well separated from interfering peaks as well as from each other (resolution ≥ 1.5; $R_t = 3.2$ and 9.9 min, respectively). Fig. 2 displays representative chromatograms of buffered extracts from blank mouse plasma, a DB-67 quality control sample (25 ng/mL of each analyte) and an experimental mouse plasma sample, which was collected 15 min after a tail vein injection of 1 mg/kg of DB-67, as described in Section 2.4.

3.3. LLOQ, linearity, accuracy and precision

Simultaneous calibration curves were generated in the ranges of 5–300 ng/mL for the carboxylate and lactone. Although initial validation attempts produced relatively linear relationships between analyte peak height and concentration ($r^2 > 0.984$), DB-67 lactone concentrations in low concentration quality control samples were consistently underestimated by 14–38% of nominal values (results not shown). The subsequent use of siliconized plastics (i.e. pipet tips and microcentrifuge tubes) in sample work-up and an increase in detector sensitivity allowed the LLOQ of both analytes to be dropped (from 5 to 1 ng/mL for the carboxylate and to 2.5 ng/mL for the lactone), increased the mean correlation coefficients of both analyte curves to $r^2 \geq 0.993$ (see Table 1), and resulted in consistent estimations of DB-67 carboxylate and lactone levels in high-, mid- and low-QC check standards within 90–110% of their nominal values (see Table 2). The variability of the slopes of the individual analyte curves was <20% throughout a 1-month period and two mobile-phase refills.

Table 2
Accuracy and precision in the quantitation of DB-67 carboxylate and lactone in spiked mouse plasma

Analyte	ng/mL	Intra-day estimates			Inter-day estimates		
		Percent of nominal value	RSD (%)	*n*	Percent of nominal value	RSD (%)	*n*
Carboxylate	25	99.0	2.9	10	99.9	1.9	5
	150	101.5	1.6	10	101.2	2.0	5
	250	95.8	5.7	10	96.6	5.4	5
Lactone	25	92.6	1.6	10	91.0	1.2	5
	150	102.7	0.9	10	101.3	2.7	5
	250	97.1	5.7	10	101.0	3.3	5

RSD: relative standard deviation.

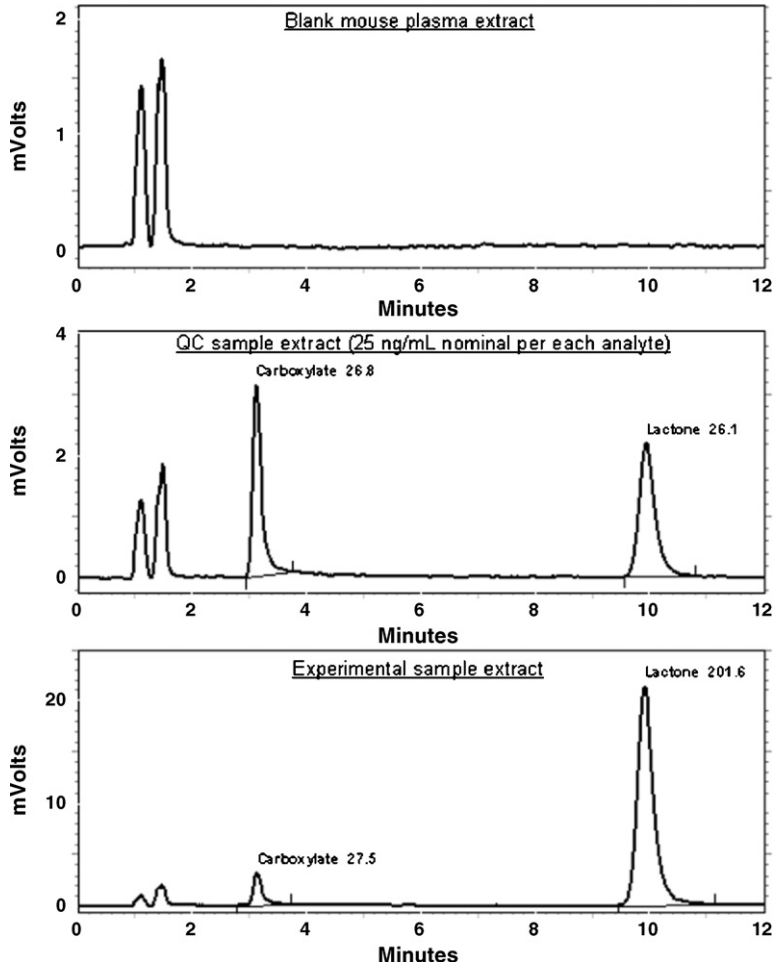


Fig. 2. HPLC chromatograms of buffer diluted methanol extracts from a blank mouse plasma sample (top trace), a 25 ng/mL DB-67 carboxylate and lactone quality control sample (middle trace), and an experimental mouse plasma sample collected 15 min after DB-67 administration (bottom trace). Measured concentrations of carboxylate and lactone within chromatographic traces are in ng/mL.

As detailed in Table 2, the assay exhibits acceptable levels of accuracy for estimating analyte concentrations (90–110% of nominal value) with percent relative standard deviation (RSD) <6%. Similar levels of accuracy were obtained in the analysis of four blind unknown sample extracts, as the percent recovery in these samples was 103.89% (SD = 11.7, RSD = 11%, $n = 4$) for the carboxylate and 110.9% (SD = 4.5, RSD = 4%, $n = 4$) for the lactone.

3.4. Stability of analytes

The stabilities of each analyte in stock and working solutions and in methanol extracts, as well as the combined analyte stabilities in mouse plasma, were carefully assessed in order to determine appropriate sample workup times and storage conditions. In all cases, results from duplicate samples at high and low analyte concentrations (250 and 25 ng/mL) were averaged and expressed as a percent of the total initial DB-67 concentration.

DB-67 was found to be sufficiently stable in DMSO (1 mg/mL) stored at −80 °C for a period of up to 4 weeks (≥93% recovery of both analytes at both QC levels, $n = 2$). A 1 h equilibration period of acidic or basic working solutions prior to their use in the preparation of calibrator or QC samples, resulted in ≥98.5% conversion of DB-67 to the lactone or carboxylate form, respectively. Short-term stability data for the combined analytes in mouse plasma after storage on ice for up to 6 h are given in Table 3. The results indicate that the lactone is converted to the carboxylate in mouse plasma over time, but that <10% conversion occurs within 6 h at 0 °C. Similar results found in long-term stability testing (Table 4) indicate that experimental samples should be used within 2 weeks of collection. Freeze/thaw analysis of analyte spiked plasma suggested that plasma should only be thawed and extracted once, as a second freeze/thaw cycle resulted in a 2 and 23% decrease in carboxylate and lactone concentrations, respectively. No degradation peaks were noted within the 12 min assay run time.

The results obtained for the short-term stability of independently extracted analytes under auto-injection conditions (1:1 dilution with mobile-phase buffer at pH 6.5, 4 °C) and long-term stability of extracts under −80 °C storage are given in Tables 3 and 4. Conversion of the carboxylate to lactone prevents reliable analysis of buffer-diluted extracts after approximately 6 h storage at 4 °C (see Table 3), whereas storage at −80 °C

Table 3
Percent DB-67 carboxylate and lactone remaining after short-term storage under various conditions

Time (h)	Carboxylate ($n=4$)		Lactone ($n=4$)	
	Buffered extracts (4 °C; pH 6.5)	Mouse plasma (0 °C)	Buffered extracts (4 °C; pH 6.5)	Mouse plasma (0 °C)
0	100	100	100	100
1	98.5 (±0.1)	102.7 (±1.3)	98.2 (±2.0)	97.0 (±0.3)
3	95.7 (±0.1)	101.6 (±1.4)	97.2 (±2.0)	98.3 (±0.9)
6	92.6 (±0.3)	102.3 (±2.3)	96.7 (±2.6)	97.3 (±0.03)

Results for high (250 ng/mL) and low (25 ng/mL) concentrations of carboxylate or lactone were combined. Values are expressed as the average percent (±standard deviation) remaining at each time point, with time zero values taken as 100%.

Table 4
Percent DB-67 carboxylate and lactone remaining after long-term storage under various conditions

Time (weeks)	Carboxylate ($n=4$)		Lactone ($n=4$)	
	MeOH extracts (−80 °C)	Mouse plasma (−80 °C)	MeOH extracts (−80 °C)	Mouse plasma (−80 °C)
0	100	100	100	100
1	98.3 (±0.2)	104.5 (±3.6)	98.2 (±1.9)	95.0 (±1.2)
2	99.7 (±6.5)	104.9 (±1.5)	100.1 (±0.1)	94.5 (±1.6)
4	100.4 (±3.9)	99.4 (±3.6)	99.9 (±0.1)	100.8 (±1.2)

Results for high (250 ng/mL) and low (25 ng/mL) concentrations of carboxylate or lactone were combined. Values are expressed as the average percent (±standard deviation) remaining at each time point, with time zero values taken as 100%.

essentially protects extracted analytes from degradation or conversion for at least 4 weeks (Table 4).

3.5. Recovery and analyte inter-conversion

Average recoveries of the carboxylate and the more highly lipophilic lactone form from mouse plasma were 100.3 and 52.4%, respectively. The effect of buffering samples prior to analysis was investigated independently for each analyte in mouse plasma extract, acid/base extract, unextracted acid/base, and in the same samples not diluted with mobile-phase buffer. The carboxylate was found to undergo an average 3.7% conversion to the lactone as a result of sample buffering prior to analysis. The lactone however did not undergo any significant inter-conversion to carboxylate in buffered as compared to unbuffered samples (<0.35%).

3.6. Pharmacokinetic analyses

The validated assay was used to determine the plasma pharmacokinetic profiles of DB-67 carboxylate and lactone in male C57BL/6 mice after intravenous administration of DB-67 (1 mg/kg). The results from this analysis and computer fits of the data to a 2-compartment model using WinNonlin are shown in Fig. 3.

3.7. Applicability of method in a pharmacokinetic study of DB-67-AB, a prodrug of DB-67

The validated method described above was further evaluated for its applicability in analyzing plasma concentrations of DB-67 lactone and carboxylate, resulting from DB-67-AB prodrug hydrolysis, along with plasma concentration of the intact prodrug. Working solutions of 50, 5, and 0.5 μg/mL DB-67-AB in 0.005 N HCl, were prepared from 1 mg/mL stock solutions of the prodrug in DMSO, and were used to spike mouse plasma samples, as described for DB-67 in Section 2. DB-67-AB eluted at 2.7 min, distinct from any interfering peaks in the matrix and with adequate resolution from the carboxylate ($R=1.0$), see Fig. 4. Calibration curves for DB-67-AB generated in the range of 1–500 ng/mL were linear ($y=0.020793x+0.43$; average $r^2=0.998$; slope RSD ≤ 12.1%; $n=4$) and accurate in predicting DB-67-AB concentrations in 250 and 2.5 ng/mL quality control samples (inter-day and intra-day mean concentrations within 96–104% of nominal values; RSD ≤ 6.8%) and in blind unknowns (mean concentrations within 94.5–105.5% of nominal values; $n=4$). Further quantitative analyses of DB-67-AB in plasma samples taken from tumor bearing mice treated with DB-67-AB (IV; 1 mg/kg) were also completed, see Fig. 5, to demonstrate the applicability of the method for more comprehensive pharmacokinetic studies to be conducted in the future.

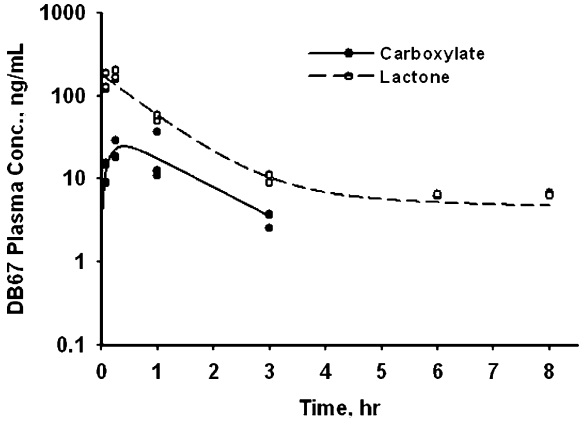


Fig. 3. Plasma concentrations of DB-67 carboxylate (●) and lactone (◯) vs. time after IV (bolus) administration of DB-67 (1 mg/kg) to C57BL/6 mice.

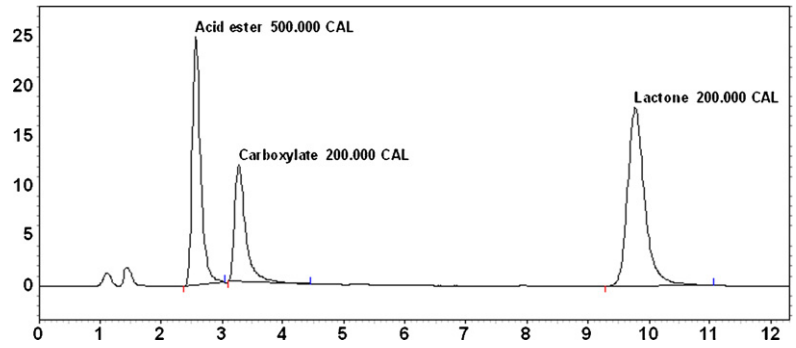


Fig. 4. Chromatographic trace of a buffered extract from mouse plasma calibrator sample containing 500 ng/mL DB-67-AB, 200 ng/mL DB-67 carboxylate and 200 ng/mL DB-67 lactone.

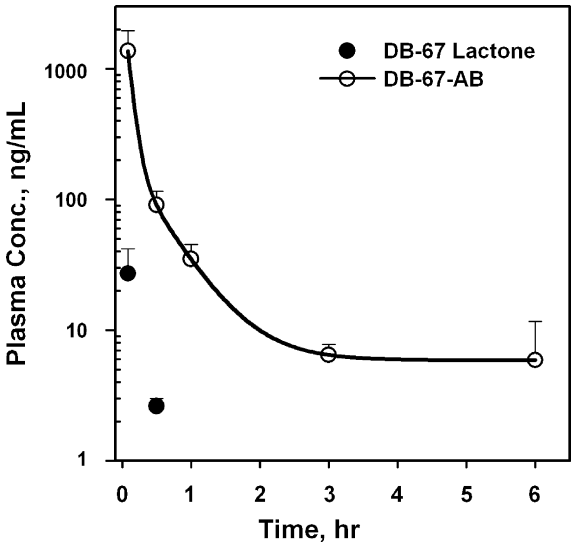


Fig. 5. DB-67-AB (◯) and DB-67 lactone (●) concentrations vs. time in plasma from tumor-bearing CB17/ICR:HSD-SCID mice treated IV with 1 mg/kg of DB-67-AB (error bars represent standard deviation resulting from $n = 3$, at all time points).

4. Discussion

An HPLC analytical method has been developed to support future studies requiring quantification of DB-67 lactone and carboxylate forms. The method involves reversed-phase chromatography for separation of the DB-67 lactone from matrix components coupled with ion-pairing reagent for the retention of the polar carboxylate. The mobile-phase content was optimized for lactone elution time as well as carboxylate peak shape and selectivity. The highly lipophilic nature of the lactone of DB-67 resulted in non-specific binding to container surfaces which necessitated the use of siliconized plastics throughout processing. Low recoveries for the lactone in plasma extracts (52.4%) may reflect extensive plasma protein binding. Nevertheless, validation efforts indicate that the assay is quite satisfactory for quantitation of DB-67 carboxylate and lactone by interpolation from linearily regressed and weighted calibration lines (range of 1–300 ng/mL and 2.5–300 ng/mL, respectively). The overall accuracy of the assay for estimating analyte concentrations in plasma samples containing 25, 150, and 250 ng/mL of each analyte was determined to be within 9% of nominal values with a relative standard deviation ≤6%.

The method was also tested for its ability to support the quantitative analysis of a potential DB-67 prodrug, DB-67-AB, in mouse plasma. The assay was found to be sufficiently accurate (within <5.5% of nominal values) and precise (RSD ≤ 6.8%) to be used in pharmacokinetic studies for the analysis of prodrug disappearance and DB-67 lactone and carboxylate formation and clearance. Additionally, given the sensitivity of analyte retention time to the mobile-phase strength, the resolution of DB-67-AB and other potential prodrugs from the DB-67 carboxylate may be readily increased by decreasing the percentage of organic solvent in the mobile phase.

References

[1] Y.H. Hsiang, L.F. Liu, Cancer Res. 48 (1988) 1722.
[2] B.C. Giovanella, J.S. Stehlin, M.E. Wall, M.C. Wani, A.W. Nicholas, L.F. Liu, R. Silber, M. Potmesil, Science 246 (1989) 1046.
[3] D. Kessel, H.B. Bosmann, K. Lohr, Biochim. Biophys. Acta 269 (1972) 210.
[4] L.H. Li, T.J. Fraser, E.J. Olin, B.K. Bhuyan, Cancer Res. 32 (1972) 2643.
[5] Y.H. Hsiang, R. Hertzberg, S. Hecht, L.F. Liu, J. Biol. Chem. 260 (1985) 14873.
[6] J. Thompson, C.F. Stewart, P.J. Houghton, Biochim. Biophys. Acta 1400 (1998) 301.
[7] C. Jaxel, K.W. Kohn, M.C. Wani, M.E. Wall, Y. Pommier, Cancer Res. 49 (1989) 1465.
[8] R.P. Hertzberg, M.J. Caranfa, K.G. Holden, D.R. Jakas, G. Gallagher, M.R. Mattern, S.M. Mong, J.O. Bartus, R.K. Johnson, W.D. Kingsbury, J. Med. Chem. 32 (1989) 715.
[9] Z. Mi, T.G. Burke, Biochemistry 33 (1994) 12540.
[10] T.G. Burke, D. Bom, Ann. N. Y. Acad. Sci. 922 (2000) 36.
[11] M. Potmesil, Cancer Res. 54 (1994) 1431.
[12] D. Bom, D.P. Curran, S. Kruszewski, S.G. Zimmer, J. Thompson Strode, G. Kohlhagen, W. Du, A.J. Chavan, K.A. Fraley, A.L. Bingcang, L.J. Latus, Y. Pommier, T.G. Burke, J. Med. Chem. 43 (2000) 3970.
[13] D.P. Curran, H. Josien, D. Bom, A.E. Gabarda, W. Du, Ann. N. Y. Acad. Sci. 922 (2000) 112.
[14] O. Lavergne, L. Lesueur-Ginot, F. Pla Rodas, D.C.H. Bigg, Bioorg. Med. Chem. Lett. 7 (1997) 2235.
[15] D. Bom, D.P. Curran, J. Zhang, S.G. Zimmer, R. Bevins, S. Kruszewski, J.N. Howe, A. Bingcang, L.J. Latus, T.G. Burke, J. Controlled Release 74 (2001) 325.
[16] X. Liu, B.C. Lynn, J. Zhang, L. Song, D. Bom, W. Du, D.P. Curran, T.G. Burke, J. Am. Chem. Soc. 124 (2002) 7650.

[17] D.L. Warner, T.G. Burke, J. Chromatogr. B: Biomed. Sci. Appl. 691 (1997) 161.
[18] K. Sano, M. Yoshikawa, S. Hayasaka, K. Satake, Y. Ikegami, H. Yoshida, T. Ishikawa, S. Sawada, S. Tanabe, J. Chromatogr. B 795 (2003) 25.
[19] A.M. Vali, B. Shafaghi, S. Dadashzadeh, J. Chromatogr. B 818 (2005) 205.
[20] X. Yang, Z. Hu, S.Y. Chan, B.C. Goh, W. Duan, E. Chan, S. Zhou, J. Chromatogr. B 821 (2005) 221.
[21] M. Leggas, Y. Zhuang, J. Welden, Z. Self, C.M. Waters, C.F. Stewart, J. Pharm. Sci. 93 (2004) 2284.
[22] T.S. Owens, H. Dodds, K. Fricke, S.K. Hanna, K.R. Crews, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 788 (2003) 65.
[23] T.X. Xiang, B.D. Anderson, Pharm. Res. 19 (2002) 1215.
[24] M. Leggas, C.F. Stewart, M.H. Woo, M. Fouladi, P.J. Cheshire, J.K. Peterson, H.S. Friedman, C. Billups, P.J. Houghton, Clin. Cancer Res. 8 (2002) 3000.
[25] Food and Drug Administration, Guidance for Industry: Bioanalytical Method Validation, 2001, http://www.fda.gov/cder/guidance/4252fnl.pdf.