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An HPLC Assay for the Lipophilic Camptothecin Analog AR-67 Carboxylate and Lactone in Human Whole Blood

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Abstract

AR-67 (7-t-butyldimethylsilyl-10-hydroxycamptothecin, DB-67) is a camptothecin analog currently in early stage clinical trials. The lactone moiety of camptothecins hydrolyzes readily in blood to yield the pharmacologically inactive carboxylate form. However the lactone form of third generation lipophilic congeners, such as AR-67, is more stable possibly due to partitioning into red cell membranes. This prompted us to develop a reverse phase HPLC method with fluorescence detection (excitation 380 nm / emission 560 nm), which could quantitate the concentration of AR-67 lactone and carboxylate in whole blood. Samples were prepared by red cell lysis, protein precipitation with methanol and centrifugation to remove denatured materials. Recovery was estimated to be >85%. Analytes were eluted isocratically with 0.15 M ammonium acetate buffer containing 10 mM TBAP (pH 6.5) and acetonitrile (65:35, v/v) on a Nova-Pak C18 column (4 μ m; 3.9 mm $\times$ 150 mm). The assay was linear in the range of 0.5–300 ng/mL and 2.5–300 ng/mL for carboxylate and lactone, respectively. Accuracy and precision were acceptable. AR-67 forms were stable in whole blood and in methanolic supernatants. This assay has been successfully applied to measure AR-67 concentrations in whole blood of patients enrolled in a phase I study.

Keywords

AR-67; HPLC; validation; whole blood

INTRODUCTION

AR-67 is a novel 3rd generation camptothecin analog currently under clinical investigation for the treatment of cancer (Figure 1). Camptothecin analogs are anticancer agents that prevent relaxation of supercoiled DNA by interacting and “poisoning” the function of topoisomerase-I. During DNA transcription and replication, camptothecins can interfere with topoisomerase-I as it tries to religate nicked DNA strands. This ultimately leads to double strand DNA breaks and cell death (Hertzberg et al., 1989; Hsiang et al., 1989; Jaxel et al., 1989). In vitro and in vivo pharmacological studies have demonstrated that the E-ring lactone and 20-hydroxyl group of camptothecin and its analogs play a fundamental role in the topoisomerase-I mediated anticancer activity (Giovannella et al., 2000; Oguma, 2001; Li et al., 2006).

One limitation to the anticancer activity of all camptothecins is the labile nature of their α -hydroxy- δ -lactone ring (lactone form), which is reversibly hydrolyzed to yield a more hydrophilic and pharmacologically inactive carboxylate at physiological pH. Lactone hydrolysis is facilitated further by favorable protein binding of the carboxylate to the human

serum albumin (HSA), which provides sink conditions and shifts the equilibrium toward carboxylate formation (Burke and Mi, 1993; Mi and Burke, 1994).

To overcome this limitation, 2nd generation analogues were designed with substituent groups that decreased the lactone hydrolysis rates. Compared to camptothecin, the clinically available analogs topotecan and irinotecan as well as its active metabolite SN-38, bind to a lesser extent to HSA and the pharmacophore lactone ring is stabilized (Burke and Mi, 1994; Mi et al., 1995). However, the clinically reported ratios of lactone areas under the concentration-time curve (AUC) for those and other congeners in early clinical development are in the order of 30-70% of the total AUC (Rothenberg et al., 1998; Zamboni et al., 1999; Crews et al., 2002; van Riel et al., 2002).

More recent efforts have focused on the development of highly lipophilic analogs, including AR-67, which have various silyl-hydrocarbon substituents that appear to minimize interaction with albumin and decrease hydrolysis rates (Bom et al., 1999; Keir et al., 2001; De Cesare et al., 2004; Sessa et al., 2007). We have previously developed an analytical method for measuring AR-67 in plasma (Horn et al., 2006). However, given its increased lipophilicity it is possible that in clinical situations AR-67 could partition into erythrocytes, which could effectively act as a drug depot (Loos et al., 2003). This has led us to develop an assay for measuring AR-67 concentration in whole blood so that we can explore relationships between pharmacokinetic parameters obtained from blood and plasma. Strong correlations between such parameters would allow the use of a blood assay and minimize the methodological efforts associated with plasma extraction and procedures designed to ensure lactone stabilization at the bedside. In this study we sought to develop processing and analytical methods that would accurately quantitate the lactone and carboxylate forms of AR-67 in whole blood. This assay would be used to analyze samples obtained from the first-in-man clinical study of AR-67.

EXPERIMENTAL

Chemicals and Reagents

Solvents including methanol and acetonitrile (from Mallinckrodt Baker, Phillipsburg, NJ) were of HPLC grade and obtained from VWR (West Chester, PA). Ammonium acetate (Mallinckrodt Baker, Phillipsburg, NJ) and sodium hydroxide (EM Science, Gibbstown, NJ) were purchased from VWR (West Chester, PA). Tetrabutylammonium dihydrogenphosphate (TBAP; 1.0 M aqueous solution) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO). Hydrochloric acid came from Fisher Scientific (Fair Lawn, NJ). Magnesium- and calcium-free Dulbecco's phosphate buffer saline (PBS) was from Gibco (Invitrogen Corp., Carlsbad, CA). Amber siliconized polypropylene micro centrifuge tubes were from Crystalgen Inc. (Plainview, NJ) and siliconized pipet tips were from VWR (West Chester, PA). AR-67 (7-tbutyldimethylsilyl-10-hydroxycamptothecin, DB-67) of high purity (>98%) was obtained from Novartis Pharmaceuticals Corporation. The solid compound was stored at -80°C. Blank human whole blood used during the validation procedure was obtained from the Central Kentucky Blood Bank (Lexington, KY), or from consenting patients enrolled in an AR-67 phase I clinical trial (Arnold et al., 2010).

Instrumentation and Chromatographic Conditions

Analysis was performed on a Shimadzu HPLC system (Shimadzu Inc., Atlanta, GA) controlled by Class-VP integrating software (Version 7.2.1). The system was equipped with an DGU-14A in-line degasser, a LC-10AD-_{VP} pump, a Shimadzu SIL-10AD-_{VP} refrigerated autoinjector with rack temperature stable at 4°C and an RF-10_{XL} fluorescence detector. Separation of compounds was carried out at ambient temperature using a reverse-phase C18 analytical column (Waters Nova-Pak C18 4 µm; 3.9 mm × 150 mm) and a C18 guard column (Waters

Nova-Pak C18 4 μ m; 3.9 mm $\times$ 20 mm). The mobile phase consisted of 0.15 M ammonium acetate buffer containing 10 mM TBAP (pH 6.5) and acetonitrile (65:35, v/v). These buffer concentrations were necessary to obtain optimal peak shape for the carboxylate analyte. In all analyses, the mobile phase was pumped at a flow rate of 1 mL/min, a 50- μ L aliquot was injected onto the chromatographic system and the excitation wavelength was set at 380 nm while the emission wavelength was set at 560 nm (Bom et al., 2001). Unless otherwise indicated, sample extracts were diluted with an equivalent volume of 0.15 M ammonium acetate buffer containing 10 mM TBAP (pH 6.5) prior to 50- μ L injection. Once stability was characterized, all buffered diluted samples were analyzed within a 6-hour window to minimize analyte interconversion.

Validation Procedures

The method was validated according to the FDA "Guidance for Industry: Bioanalytical Method Validation" document ((FDA), 2001). Certain aspects of the validation process are presented below.

Specificity and Selectivity

Whole blood samples from four different sources were spiked (0.5 - 5 ng/mL) with either lactone or carboxylate and were injected in triplicate. The lowest limit of quantification (LLOQ) was determined based upon analyte signal to noise ratio of ≥ 5 , analyte accuracy of 80-120%, and precision of $< 20\%$.

Calibration, Quality Control and Experimental Sample Preparation

During the preparation of calibrators and control samples, all aqueous diluents and solutions were kept on wet ice and all methanolic diluents and solutions were kept on dry ice. Amber containers were used throughout sample preparation to avoid photo degradation while siliconized plastics and glass were used to prevent drug absorption. An accurately weighed amount of AR-67 was dissolved in DMSO to produce a stock solution of 1 mg/mL that was aliquoted (30 μ L) for single-time use. These aliquots were stored at -80°C for up to one month (Horn et al., 2006). Aqueous working solutions of 10, 1 and 0.1 $\mu\text{g/mL}$ were freshly prepared from stock in 0.005 N sodium hydroxide solution or 0.005 N hydrochloric acid solutions for carboxylate or lactone, respectively. The carboxylate working solutions were prepared by serial dilutions whereas the lactone working solutions were prepared individually from the stock solution. Working solutions were equilibrated for one hour on ice (4°C). Whole blood calibrators containing 0, 0.5, 1, 5, 10, 20, 50, 100, 200 and 300 ng/mL of AR-67 carboxylate and 0, 2.5, 5, 10, 20, 50, 100, 200 and 300 ng/mL of AR-67 lactone were prepared by adding the appropriate volumes of the individual working solutions to 940 μ L of whole blood and diluted to 1000 μ L with 1x PBS (pH 7.4). The lactone form of AR-67 was generally added prior to the carboxylate form. After vortex mixing for 10 sec, the spiked whole blood samples were frozen at -80°C for 30 min to lyse the blood cells. Lysed samples were thawed at room temperature and deproteinated with 4 volumes of cold (-80°C) methanol. The methanolic mixture was vortexed for 10 min at room temperature and centrifuged at $13,000 \times g$ (10 min, 4°C). The methanolic supernatants were decanted into amber tubes and analyzed immediately as indicated above or stored at -80°C until analysis. Finally, the methanolic extracts were diluted 1:1 with mobile phase buffer before being placed in the Shimadzu SIL-10AD.VP refrigerated autoinjector to be injected. Three quality control samples (QC) containing 1.5 ng/mL of carboxylate and 7.0 ng/mL of lactone (QC1), 150 ng/mL of each analyte (QC2) and 250 ng/mL of each analyte (QC3) were prepared following the same procedure as described for the calibrators. Quality control methanolic supernatants were used in assay validation and to confirm daily system suitability throughout the analysis of experimental samples. The acceptance criterion for each back-calculated quality control sample concentration was 15% deviation from the nominal.

Interconversion and Stability Experiments

Experiments were conducted to compare the interconversion of analytes in individually spiked whole blood samples (250 ng/mL carboxylate or lactone) and in purely basic (0.005 N NaOH) and acidic (0.005 N HCl) solutions diluted as necessary (250 ng/mL carboxylate or lactone).

Stability tests were performed on QC1 and QC3 whole blood samples and their methanolic supernatants. Samples were deemed stable if their assayed concentration remained within 15% of the initially measured concentration. When short term stability of methanolic supernatants was studied, either lactone or carboxylate supernatants were stored in the autosampler (4°C) for up to 24 hours after being diluted with an equal volume of mobile phase buffer. Samples were injected at 0, 1, 3, 6 and 24 hours and analytical HPLC runs were conducted based on the results regarding the stability of the methanolic extracts in mobile phase buffer. For long term stability of the methanolic supernatants, the supernatants containing both analytes were stored at -80°C and were mixed 1:1 with mobile phase buffer immediately before injection at predetermined time points (0, 1, 5 days, 1 and 2 weeks, 1 and 2 months). Moreover, short (0, 1, 3 and 6 hours at 4°C) and long term (0, 1 and 3 days, 1 and 2 weeks, 1 and 2 months at -80°C) stability was evaluated in human whole blood. In those cases, the analytes were extracted from QC samples at the experimental time points and injected after being mixed 1:1 with mobile phase buffer. Single injections of triplicate samples were analyzed unless otherwise indicated.

Recovery

Single injections of triplicate QC1 and QC3 methanolic supernatants were used to evaluate the recovery of both AR-67 forms. Recovery was estimated by comparing peak heights of whole blood methanolic supernatants to those of carboxylate and lactone methanol solutions freshly prepared and immediately injected after dilution with an equal volume of mobile phase buffer.

Statistical Analysis

Data and regression analyses were performed with Shimadzu Class VP software (Version 7.2.1.). A linear regression model was used to fit the peak heights from the calibration solutions. Inverse or inverse square weighting schemes were used as needed to increase the correlation coefficient. Control and unknown sample concentrations were calculated using the resulting standard curve equations.

Pharmacokinetics in Humans

The pharmacokinetic profile of AR-67 in whole blood samples was obtained from a consenting patient enrolled in a phase I clinical trial. The study was approved by institutional review boards and met all ethical and research standards. Design of that trial will be reported elsewhere (Arnold et al., 2010). The patient received a dose of 6.3 mg/m² AR-67 as an 1-hour intravenous infusion and blood samples were collected at 0, 10, 45 minutes, 1, 1.25, 1.5, 2, 4, 6 and 8 hours after the start of the infusion. Collected blood samples were transferred into amber siliconized microcentrifuge tubes, and stored on dry ice for later transfer to -80°C freezer. Within one month from the time of collection, blood samples were thawed and processed. Methanolic supernatants were analysed within 1 week from their preparation. Once processed and diluted with buffer, samples were analyzed within a 6-hour window to minimize analyte interconversion.

RESULTS

Sample Processing and Assay Development

The conditions used for processing and quantitating AR-67 from human whole blood were adapted and developed from those used to quantify AR-67 in mouse plasma (Horn et al.,

2006). Due to the lipophilicity of the AR-67 lactone, we expected increased partitioning in red blood cell membranes. Thus, a lysis step was implemented to increase lactone recovery. In addition, the deproteinization time was increased from 10 sec to 10 min, while vortex mixing, and the resulting solution was centrifuged at 13,000x rpm for 10 min, rather than 2 min, in order to ensure complete removal of particulates.

Selectivity and Specificity

The carboxylate and lactone of AR-67 were well separated from interfering matrix peaks and from each other (resolution ≥ 1.5 ; $R_t = 3.2$ and 9.7 min for carboxylate and lactone, respectively) at all concentrations. Four different human whole blood sources were analyzed and endogenous components did not interfere with any of the analytes at analyte LLOQ values (Figure 2).

Linearity, LLOQ, Accuracy and Precision

Calibration curves prepared in whole blood were created in the range of 0.5-300 ng/mL for the carboxylate and 2.5-300 ng/mL for the lactone moiety. As shown in Table 1, three calibration curves were prepared from different healthy human volunteer whole blood sources, resulting in a mean correlation coefficient for both analyte curves of $r^2 \geq 0.999$. The linearity between the detector response and the nominal concentration of the analytes, allows for accurate and precise estimations of AR-67 carboxylate and lactone concentrations in high, mid, and low quality control solutions, which were within 85-115% of the expected concentration value (Table 1). The LLOQ for carboxylate was determined to be 0.5 ng/mL while for lactone was found to be 2.5 ng/mL. The reproducibility of the analytical method was evaluated by calculating the intra- and interday relative standard deviations (RSDs). The intraday variability for low, mid, and high QC concentrations was $<5.2\%$ and $<3.4\%$ for carboxylate and lactone, respectively. The interday variability for low, mid, and high QC concentrations was $<8.4\%$ and 9.3% for carboxylate and lactone respectively. These results indicate that the assay method is reproducible within the same day as well as on different days (Table 1).

Interconversion and Stability of Analytes

Experiments to determine the interconversion of analytes in whole blood spiked individually with 250 ng/mL carboxylate or lactone indicated that sample workup resulted in 6.1% conversion of carboxylate to lactone and 2.2% conversion of lactone to carboxylate ($n=2-5$). Analytes prepared in basic (pH 11.3; 0.005 N NaOH) and acidic (pH 3.4; 0.005 N HCl) solutions and processed the same as the whole blood samples yielded no interconversion for either analyte.

Short term stability of methanolic supernatants indicated that lactone and carboxylate, in low and high concentration (QC1 and QC3), were stable for at least 6 hours in the autoinjector (4°C) after being and diluted (1:1, v/v) with mobile phase buffer (Table 2). Long term stability of the methanolic supernatants indicated that they were stable for up to 14 days if stored at -80°C (Table 3). Short term stability in whole blood indicated that analytes were stable for 6 hours at 4°C (Table 2). The lactone form of AR-67 in spiked whole blood samples proved to be stable after being stored at -80°C for at least 28 days. However, the carboxylate concentrations decreased to approximately 85% of the initial values by 1 week in storage at -80°C , but further decrease was not observed by the end of week 4 (Table 3). Freeze-thaw analysis of whole blood samples spiked with low (QC1) or high (QC3) concentrations of either lactone or carboxylate were stable after three freeze-thaw cycles (Table 4).

Recovery

Recovery was similar at low and high concentrations of each analyte (Table 4). In whole blood samples spiked with low concentrations (QC1), average recovery was 94.8% (± 3.0) and

109.7% (± 5.3) for carboxylate and lactone, respectively. Recovery in samples spiked with high concentrations (QC3), average recovery was 86.6% (± 1.0) and 99.8% (± 1.4) for carboxylate and lactone, respectively.

Clinical application

This method enabled us to determine the pharmacokinetic profile of AR-67 following intravenous administration to patients with solid malignancies. Blood concentrations for AR-67 carboxylate and lactone versus time are shown in Figure 3 for one of the patients participating in the study.

DISCUSSION

An assay to quantitate carboxylate and lactone AR-67 forms in human whole blood has been developed and validated. The analysis was performed using reversed-phase high performance liquid chromatography coupled with fluorescence detection. Sample processing included a red cell lysis step by storage on dry ice or -80°C to ensure efficient recovery of both the hydrophilic carboxylate and, in particular, the highly lipophilic lactone, followed by a simple methanolic protein precipitation step.

Although numerous analytical methods using HPLC (de Jong et al., 2003) ; Horn et al., 2006 ; Hubbard et al., 2009) have been developed for the quantification of camptothecin analogs in biological matrices, the challenge of their chemical instability remains. Our method demonstrates that protein precipitation with cold (-80°C) methanol efficiently releases protein bound analytes and provides adequate stability for accurate analysis in the laboratory setting, provided that methanolic supernatants are stored at -80°C and analyzed at 4°C within 2 weeks of sample processing. The present assay allows for the accurate and precise quantitation of AR-67 carboxylate and lactone forms in human whole blood making it a valid assay for future clinical studies. Its linearity in the range of 0.5-300 ng/mL for the carboxylate and 2.5-300 ng/mL for the lactone was found satisfactory and allows quantitation of both analytes in biological samples within this concentration range. In contrast to other methods, acidification of the blood samples is not necessary since complete separation between both analytes and endogenous compounds was achieved under the chromatographic conditions described above. Red cell lysis followed by the methanolic protein precipitation appears to efficiently recover both the carboxylate and lactone from the biological matrix. The LLOQ for carboxylate and lactone (0.5 and 2.5 ng/mL, respectively) allows for its application to future pharmacokinetic studies. Stability tests have shown that both AR-67 forms are stable after 3 freeze-thaw cycles. Most importantly, although both carboxylate and lactone forms are stable in their methanolic extracts for up to two weeks, they seem to be stable in whole blood for a longer period. The lactone form appears to be more stable in the biological matrix compared to the carboxylate form. This apparent stability could also be because the highly lipophilic lactone may absorb onto surfaces but is replenished via conversion from the carboxylate. This was prevented to a large extent by pretreating all consumables with a siliconizing solution. However, absorption of the highly lipophilic lactone may continue, albeit at a much reduced rate, over time. Under our processing and chromatography conditions, analysis of experimental samples with high lactone concentrations is likely to overestimate the carboxylate concentration. However, given the relatively high lactone concentrations (Figure 3), this is insignificant in relative terms. Previous clinical work has demonstrated that camptothecins interact with red cells. It has been reported that irinotecan is selectively partitions into red blood cells, while its metabolite SN-38 distributes preferentially in the plasma compartment (de Jong et al., 2003). Interestingly, differences on the pharmacokinetic profile of topotecan between the two sexes were noted, but ultimately they were attributed to hematocrit differences between males and females (Gallo et al., 2000; Loos et al., 2003; Hubbard et al., 2009). Finally, studies have shown that red blood

cells serve as a depot compartment for the lactone 9-amino-20(S)-camptothecin (Loos et al., 1999). Consequently, pharmacokinetic parameters such as the volume of distribution and the clearance depend greatly on the biological matrix from which the sample was collected (Peck et al., 1992). The development of the current analytical method for assaying AR-67 in whole blood will allow us to study the pharmacokinetics of AR-67 in the blood compartment.

The assay was successfully applied to determine the concentrations of AR-67 in cancer patients enrolled in a phase I clinical trial (Arnold et al., 2010). As demonstrated in Figure 3, the lactone concentration was higher than the carboxylate at all time points. This was consistent with the expected behavior of the lactone partitioning in red cell membranes. Interestingly, the pharmacokinetic profiles of AR-67 in blood and in plasma appeared to mirror each other indicating that the red cells were not acting as a depot for AR-67 (data not shown). Further studies are in progress to assess whether patient dependent covariates, such as hematocrit, age and weight play a role in the disposition of AR-67.

CONCLUSIONS

A high-performance liquid chromatography method for simultaneous determination of AR-67 carboxylate and lactone in whole blood has been developed and validated. The method has been shown to be sensitive, accurate and precise. Lactone stability during sample processing remains a concern if temperature is not controlled and consumables are not siliconized, but AR-67 is stable in whole blood stored under the proper conditions (-80°C). This is the first reported method for the analysis of AR-67 in whole blood matrix and will be used for the analysis of samples obtained from patients treated in clinical studies.

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References

- FDA Guidance for Industry Bioanalytical Method Validation.. US Food and Drug Administration Center for Evaluation and Research Website. [Sept 13,2004]. Available from: URL: <http://www.fda.gov/cder/guidance/index.htm>.
- Arnold S, Rinehart J, Tsakalozou E, Eckardt J, Fields S, Shelton B, DeSimone P, Kee B, Moscow J, Leggas M. A phase I study of 7-t-Butyldimethylsilyl-10-Hydroxycamptothecin in adult patients with refractory or metastatic solid malignancies. Clin Cancer Res. 2010 doi: 10.1158/1078-0432.CCR-09-2429.
- Bom D, Curran DP, Chavan AJ, Kruszewski S, Zimmer SG, Fraley KA, Burke TG. Novel A,B,E-ring-modified camptothecins displaying high lipophilicity and markedly improved human blood stabilities. J Med Chem 1999;42:3018–3022. [PubMed: 10447944]
- Bom D, Curran DP, Zhang J, Zimmer SG, Bevins R, Kruszewski S, Howe JN, Bingcang A, Latus LJ, Burke TG. The highly lipophilic DNA topoisomerase I inhibitor DB-67 displays elevated lactone levels in human blood and potent anticancer activity. Journal of Controlled Release 2001;74:325–333. [PubMed: 11489514]
- Burke TG, Mi Z. Preferential binding of the carboxylate form of camptothecin by human serum albumin. Anal Biochem 1993;212:285–287. [PubMed: 8368506]
- Burke TG, Mi Z. The structural basis of camptothecin interactions with human serum albumin: impact on drug stability. J Med Chem 1994;37:40–46. [PubMed: 8289200]
- Crews KR, Stewart CF, Jones-Wallace D, Thompson SJ, Houghton PJ, Heideman RL, Fouladi M, Bowers DC, Chintagumpala MM, Gajjar A. Altered Irinotecan Pharmacokinetics in Pediatric High-Grade Glioma Patients Receiving Enzyme-inducing Anticonvulsant Therapy. Clin Cancer Res 2002;8:2202–2209. [PubMed: 12114421]

- De Cesare M, Pratesi G, Veneroni S, Bergottini R, Zunino F. Efficacy of the Novel Camptothecin Gimitecan against Orthotopic and Metastatic Human Tumor Xenograft Models. *Clin Cancer Res* 2004;10:7357–7364. [PubMed: 15534112]
- de Jong FA, Mathijssen RH, de Bruijn P, Loos WJ, Verweij J, Sparreboom A. Determination of irinotecan (CPT-11) and SN-38 in human whole blood and red blood cells by liquid chromatography with fluorescence detection. *J Chromatogr B Analyt Technol Biomed Life Sci* 2003;795:383–388.
- Gallo JM, Laub PB, Rowinsky EK, Grochow LB, Baker SD. Population Pharmacokinetic Model for Topotecan Derived From Phase I Clinical Trials. *J Clin Oncol* 2000;18:2459–2467. [PubMed: 10856106]
- Giovannella BC, Harris N, Mendoza J, Cao Z, Liehr J, Stehlin JS. Dependence of anticancer activity of camptothecins on maintaining their lactone function. *Ann N Y Acad Sci* 2000;922:27–35. [PubMed: 11193902]
- Hertzberg RP, Caranfa MJ, Holden KG, Jakas DR, Gallagher G, Mattern MR, Mong SM, Bartus JO, Johnson RK, Kingsbury WD. Modification of the hydroxy lactone ring of camptothecin: inhibition of mammalian topoisomerase I and biological activity. *J Med Chem* 1989;32:715–720. [PubMed: 2537428]
- Horn J, Jordan SL, Song L, Roberts MJ, Anderson BD, Leggas M. Validation of an HPLC method for analysis of DB-67 and its water soluble prodrug in mouse plasma. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2006
- Hsiang YH, Liu LF, Wall ME, Wani MC, Nicholas AW, Manikumar G, Kirschenbaum S, Silber R, Potmesil M. DNA topoisomerase I-mediated DNA cleavage and cytotoxicity of camptothecin analogues. *Cancer Res* 1989;49:4385–4389. [PubMed: 2545341]
- Hubbard KE, Schaiquevich P, Bai F, Fraga CH, Miller L, Panetta JC, Stewart CF. Application of a highly specific and sensitive fluorescent HPLC method for topotecan lactone in whole blood. *Biomed Chromatogr*. 2009
- Jaxel C, Kohn KW, Wani MC, Wall ME, Pommier Y. Structure-activity study of the actions of camptothecin derivatives on mammalian topoisomerase I: evidence for a specific receptor site and a relation to antitumor activity. *Cancer Res* 1989;49:1465–1469. [PubMed: 2538227]
- Keir ST, Hausheer F, Lawless AA, Bigner DD, Friedman HS. Therapeutic activity of 7-[(2-trimethylsilyl)ethyl]-20 (S)-camptothecin against central nervous system tumor-derived xenografts in athymic mice. *Cancer Chemother Pharmacol* 2001;48:83–87. [PubMed: 11488529]
- Li QY, Zu YG, Shi RZ, Yao LP. Review camptothecin: current perspectives. *Curr Med Chem* 2006;13:2021–2039. [PubMed: 16842195]
- Loos WJ, Gelderblom H, Verweij J, van Boven-van Zomeren DM, Nooter K, Stoter G, Sparreboom A. Red blood cells: a neglected compartment in topotecan pharmacokinetic analysis. *Anticancer Drugs* 2003;14:227–232. [PubMed: 12634617]
- Loos WJ, Verweij J, Gelderblom HJ, de Jonge MJ, Brouwer E, Dallaire BK, Sparreboom A. Role of erythrocytes and serum proteins in the kinetic profile of total 9-amino-20(S)-camptothecin in humans. *Anticancer Drugs* 1999;10:705–710. [PubMed: 10573202]
- Mi Z, Burke TG. Differential interactions of camptothecin lactone and carboxylate forms with human blood components. *Biochemistry* 1994;33:10325–10336. [PubMed: 8068669]
- Mi Z, Malak H, Burke TG. Reduced albumin binding promotes the stability and activity of topotecan in human blood. *Biochemistry* 1995;34:13722–13728. [PubMed: 7577964]
- Oguma T. Antitumor drugs possessing topoisomerase I inhibition: applicable separation methods. *J Chromatogr B Biomed Sci Appl* 2001;764:49–58. [PubMed: 11817043]
- Peck CC, Barr WH, Benet LZ, Collins J, Desjardins RE, Furst DE, Harter JG, Levy G, Ludden T, Rodman JH, et al. Opportunities for integration of pharmacokinetics, pharmacodynamics, and toxicokinetics in rational drug development. *Pharm Res* 1992;9:826–833. [PubMed: 1409369]
- Rothenberg ML, Kuhn JG, Schaaf LJ, Drengler RL, Eckhardt SG, Villalona-Calero MA, Hammond L, Miller LL, Petit RG, Rowinsky EK, Von Hoff DD. Alternative dosing schedules for irinotecan. *Oncology (Williston Park)* 1998;12:68–71. [PubMed: 9726095]
- Sessa C, Cresta S, Cerny T, Baselga J, Rota Caremoli E, Malossi A, Hess D, Trigo J, Zucchetti M, D'Incalci M, Zaniboni A, Capri G, Gatti B, Carminati P, Zanna C, Marsoni S, Gianni L. Concerted

- escalation of dose and dosing duration in a phase I study of the oral camptothecin gimatecan (ST1481) in patients with advanced solid tumors. *Ann Oncol* 2007;18:561–568. [PubMed: 17150998]
- van Riel JMGH, van Groeningen CJ, Kedde MA, Gall H, Leisink JMA, Gruia G, Pinedo HM, van der Vijgh WJF, Giaccone G. Continuous Administration of Irinotecan by Hepatic Arterial Infusion: A Phase I and Pharmacokinetic Study. *Clin Cancer Res* 2002;8:405–412. [PubMed: 11839656]
- Zamoni WC, Bowman LC, Tan M, Santana VM, Houghton PJ, Meyer WH, Pratt CB, Heideman RL, Gajjar AJ, Pappo AS, Stewart CF. Interpatient variability in bioavailability of the intravenous formulation of topotecan given orally to children with recurrent solid tumors. *Cancer Chemother Pharmacol* 1999;43:454–460. [PubMed: 10321504]

Figure 1.
Chemical structure of the lactone and carboxylate form of the camptothecin analog, AR-67.

Figure 2. Representative chromatographic traces of methanolic supernatants generated from (A) blank human whole blood, (B) low concentration quality control sample (QC1), (C) high concentration quality control sample (QC3), and (D) phase I patient sample collected 5 min after the start of a 1-hour intravenous infusion of AR-67 lactone.

Figure 3.
Pharmacokinetic profile of AR-67 in whole blood from a patient after administration of AR-67 (6.3 mg/m^2) as an intravenous infusion for 60 min.

Table 1

Assay regression, accuracy and precision.

Analyte Calibration Curves (n=3)	Average Regression Parameters * ($\pm$ standard deviation)			
	Slope	y-intercept	R ²	Weighting
Carboxylate 0, 0.5-300 ng/mL	0.0003395 ($\pm$ 0.0000265)	0.2090 ($\pm$ 0.1490)	0.999 ($\pm$ 0.0016)	1/Amount ²
Lactone 0, 2.5-300 ng/mL	0.0005107 ($\pm$ 0.0000064)	0.4419 ($\pm$ 0.3239)	0.999 ($\pm$ 0.0002)	1/Response

Intra-day Accuracy and Precision (n=5)**	Average Percent Accuracy (Relative Standard Deviation)		
	QC-1	QC-2	QC-3
Carboxylate	99.6 (5.23 %)	105.0 (2.66 %)	104.3 (3.25 %)
Lactone	86.7 (1.26 %)	105.4 (2.13 %)	105.6 (3.43 %)

Inter-day Accuracy and Precision (n=10)**	Average Percent Accuracy (Relative Standard Deviation)		
	QC-1	QC-2	QC-3
Carboxylate	89.0 (1.87 %)	100.0 (8.36 %)	99.7 (5.92 %)
Lactone	113.7 (1.35 %)	97.5 (9.26 %)	103.2 (6.36 %)

* Regression with peak height as independent variable.

** Accuracy was measured as the percent of nominal with quality control samples containing 1.5 ng/mL carboxylate and 7 ng/mL lactone (QC-1), 150 ng/mL each analyte (QC-2), or 250 ng/mL each analyte (QC-3).

Table 2

Analyte short term stability in whole blood and in buffer diluted methanolic supernatants stored at 4°C.

Whole Blood	Average Percent Remaining at Given Time Point* (± standard deviation)				
	Time 0	1 hr	3 hr	6 hr	
QC-1 (n=3)	Carboxylate	100.0	108.5 (±6.5)	104.5 (±8.6)	104.8 (±2.9)
	Lactone	100.0	101.7 (±5.8)	97.2 (±5.2)	96.3 (±2.8)
QC-3 (n=3)	Carboxylate	100.0	99.4 (±3.8)	99.1 (±6.9)	97.8 (±4.3)
	Lactone	100.0	102.5 (±2.1)	108.1 (±5.9)	109.8 (±5.1)

Average Percent Remaining at Given Time Point (± standard deviation)						
Buffer Diluted Methanolic Supernatants	Time 0	1 hr	3 hr	6 hr	24 hr	
QC-1 (n=3)	Carboxylate	100.0	101.3 (±2.4)	99.8 (±4.4)	102.3 (±2.6)	121.9 (±6.1)
	Lactone	100.0	99.9 (±3.4)	98.3 (±3.8)	95.8 (±2.6)	95.7 (±1.5)
QC-3 (n=3)	Carboxylate	100.0	96.8 (±1.2)	95.7 (±1.6)	91.0 (±3.2)	79.7 (±1.2)
	Lactone	100.0	101.3 (±3.1)	102.4 (±1.5)	107.4 (±3.0)	113.7 (±3.1)

* Analyte levels expressed as the average percent of average time zero values with quality control samples containing 1.5 ng/mL lactone (QC-1), or 250 ng/mL each analyte (QC-3).

Table 3

Analyte long term stability in whole blood and methanolic supernatants stored at -80°C.

Whole Blood		Average Percent Remaining at Given Time Point* (± standard deviation)						
	Time 0	1 Day	3 Days	1 Week	2 Weeks	1 Month	2 Months	
QC-1 (n=3)	Carboxylate	100.0	101.11 (±6.7)	105.64 (±9.8)	99.08 (±10.0)	109.28 (±5.2)	77.49 (±3.0)	95.87 (±1.9)
	Lactone	100.0	103.41 (±2.3)	104.51 (±11.5)	98.77 (±7.8)	105.23 (±4.2)	87.57 (±1.3)	118.66 (±7.1)
QC-3 (n=3)	Carboxylate	100.0	89.03 (±8.0)	88.43 (±8.1)	85.05 (±7.1)	83.05 (±3.4)	79.95 (±5.3)	84.58 (±7.0)
	Lactone	100.0	97.64 (±8.0)	97.12 (±10.0)	84.28 (±7.9)	87.62 (±4.4)	90.74 (±6.4)	111.12 (±10.4)
Methanolic Supernatants		Average Percent Remaining at Given Time Point* (± standard deviation)						
	Time 0	1 Day	5 Days	1 Week	2 Weeks	1 Month	2 Months	
QC-1 (n=3)	Carboxylate	100.0	107.4 (±1.8)	101.2 (±1.14)	93.4 (±8.3)	93.7 (±6.6)	82.4 (±3.6)	60.0 (±9.4)
	Lactone	100.0	109.9 (±3.1)	100.3 (±2.6)	94.4 (±0.8)	96.0 (±2.4)	86.7 (±2.7)	75.0 (±3.1)
QC-3 (n=3)	Carboxylate	100.0	102.1 (±0.3)	95.6 (±1.9)	90.0 (±3.2)	86.7 (±1.5)	81.4 (±2.4)	71.8 (±2.2)
	Lactone	100.0	107.1 (±1.8)	98.9 (±2.1)	94.1 (±2.8)	93.3 (±1.7)	88.2 (±2.4)	77.5 (±1.7)

* Analyte levels expressed as the average percent of average time zero values with quality control samples containing 1.5 ng/mL lactone (QC-1), or 250 ng/mL each analyte (QC-3).

Table 4

Analyte recovery and freeze-thaw (F-T) stability.

Percent of Methanolic Analyte Solutions ($\pm$ standard deviation)				
Recovery*	QC-1 (n=3)	QC-3 (n=3)		
Carboxylate	94.76 ($\pm$ 2.99)	86.6 ($\pm$ 0.99)		
Lactone	109.66 ($\pm$ 5.30)	99.83 ($\pm$ 1.40)		

Average Percent Remaining at Given Cycle ($\pm$ standard deviation)				
Freeze-Thaw Stability**	Cycle	0	1	2
QC-1 (n=5)	Carboxylate	100	119.52 ($\pm$ 9.19)	97.73 ($\pm$ 6.32)
	Lactone	100	120.24 ($\pm$ 16.00)	109.25 ($\pm$ 21.02)
QC-3 (n=5)	Carboxylate	100	108.56 ($\pm$ 14.83)	108.67 ($\pm$ 10.28)
	Lactone	100	113.43 ($\pm$ 10.14)	115.95 ($\pm$ 11.50)

* Recovery was measured as percent peak height of individually spiked methanolic supernatants to individually spiked methanolic solutions.

** Stability given as their percentage of Cycle 0 averages.