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Development of a high-performance liquid chromatographic method to determine the concentration of karenitecin, a novel highly lipophilic camptothecin derivative, in human plasma and urine

Judith A. Smith[a], Fredrick Hausheer[c], Robert A. Newman[b], Timothy L. Madden[a,b,*]

[a]*Division of Pharmacy, University of Texas M.D. Anderson Cancer Center, Houston, TX, USA*
[b]*Pharmaceutical Development Center, Univeristy of Texas, M.D. Anderson Cancer Center, Houston, TX, USA*
[c]*BioNumerik Pharmaceuticals, Inc, San Antonio, TX, USA*

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Abstract

Karenitecin is a novel, highly lipophilic camptothecin derivative with potent anticancer potential. We have developed a sensitive high-performance liquid chromatographic method for the determination of karenitecin concentration in human plasma and urine. Karenitecin was isolated from human plasma and urine using solid-phase extraction. Separation was achieved by gradient elution, using a water and acetonitrile mobile phase, on an ODS analytical column. Karenitecin was detected using fluorescence detection at excitation and emission wavelengths of 370 and 490 nm, respectively. Retention time for karenitecin was 16.2±0.5 min and 8.0±0.2 min for camptothecin, the internal standard. The karenitecin peak was baseline resolved, with the nearest peak at 3.1 min distance. Using normal volunteer plasma and urine from multiple individuals, as well as samples from the 50 patients analyzed to date, no interfering peaks were detected. Inter- and intra-day coefficients of variance were <4.4 and 7.1% for plasma and <4.9 and 11.6% for urine. Assay precision, based on an extracted karenitecin standard plasma sample of 2.5 ng/ml, was +4.46% with a mean accuracy of 92.4%. For extracted karenitecin standard urine samples of 2.5 ng/ml assay precision was +2.35% with a mean accuracy of 99.5%. The mean recovery of karenitecin, at plasma concentrations of 1.0 and 50 ng/ml, was 81.9 and 87.8% respectively. In urine, at concentrations of 1.5 and 50 ng/ml, the mean recoveries were 90.3 and 78.4% respectively. The lower limit of detection (LLD) for karenitecin was 0.5 ng/ml in plasma and 1.0 ng/ml in urine. The lower limit of quantification (LLQ) for karenitecin was 1 ng/ml and 1.5 ng/ml for plasma and urine, respectively. Stability studies indicate that when frozen at −70°C, karenitecin is stable in human plasma for up to 3 months and in human urine for up to 1 month. This method is useful for the quantification of karenitecin in plasma and urine samples for clinical pharmacology studies in patients receiving this agent in clinical trials.

Keywords: Karenitecin; Camptothecin

1. Introduction

Karenitecin, 7-[(2-trimethysilyl) ethyl]-20(*S*)-camptothecin, is a novel, highly lipophilic camptothecin

*Corresponding author. University of Texas, M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Box 90, Houston, TX 77030, USA. Tel.: +1-713-745-1302; fax: +1-713-797-9693.
E-mail address: tmadden@mdanderson.org (T.L. Madden).

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derivative. The proposed mechanism of action for the class of camptothecins is inhibition of topoisomerase I by binding the enzyme, stabilizing the ''cleavable complex'' along the DNA replication fork which results in an accumulation of single strand and double strand DNA breaks and ultimately cell death [1,2]. More recent information suggests that the primary, highly selective camptothecin lactone target is on the DNA [4]. High-field NMR confirmed the lactone solely binds to double-strand and single-strand DNA in the absence of topoisomerase I and the binding is sequence specific [3].

Camptothecin derivatives undergo a rapid, reversible, non-enzymatic, pH dependent hydrolysis from the closed E-ring lactone form of the drug, which possesses anti-tumor activity, to the inactive hydroxy acid [1,2]. Hence, these agents have limited stability at physiologic pH, a characteristic that contributes to the variability observed in their pharmacokinetic and pharmacodynamic behavior. Karenitecin was supercomputer engineered to have more desirable physicochemical characteristics to improve its lactone stability.

Molecular alterations resulting in E-ring stabilization are hypothesized to conserve the drug in the closed-ring, active lactone form [4]. For karenitecin this E-ring stabilization was accomplished by the addition of a tri-methyl-silyl (TMS) at position 7 on ring B (Fig. 1). It has been proposed that this TMS group contributes to improved lactone stability.

Since the introduction of camptothecin derivatives into clinical drug development, a number of investigators have been faced with the challenge of accurate quantification of the highly unstable active lactone form of these agents. Nearly all of the previously published HPLC assays for camptothecins involve the use of a mobile phase containing buffer, such as ammonium acetate or phosphate buffer to achieve peak separation and minimize peak tailing [5]. Furthermore, the pH of the buffered mobile phase had to be adjusted to maintain an acidic pH due to the instability of the active lactone form at basic pH.

In this paper, we report a highly sensitive HPLC method for the determination of karenitecin in human plasma and urine, using fluorescence detection that did not necessitate the use of a buffered, pH adjusted mobile phase for separation. This allows complete quantification of the active lactone drug present in samples using a less complex mobile phase, thereby simplifying the analytical process while prolonging analytical column life.

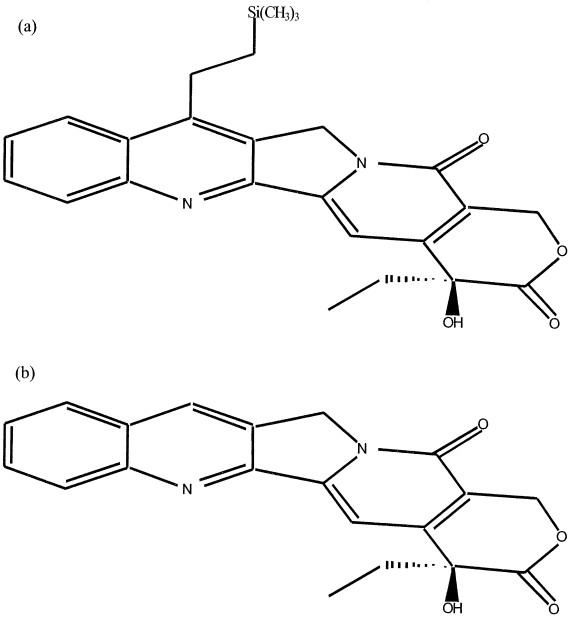

Fig. 1. (a)Structure of karenitecin (7-[(2-trimethylsilyl)ethyl]-20(*S*)-Camptothecin) MW=448.59. (b) Structure of 20(*S*)-Camptothecin MW=348.36.

2. Experimental

2.1. Chemicals and reagents

Karenitecin, 99.8% pure, was graciously provided by BioNumerik Chemical Laboratories. HPLC grade acetonitrile, dimethyl sulfoxide (DMSO) and camptothecin, used as the internal standard, were purchased from Sigma (St. Louis, MO). Purified deionized water, was produced using a Milli-Q Academic Quantum Purification System (Millipore Corporation, Bedford, MA). Eighty-five percent *O*-phosphoric acid was purchased from Fischer Scientific (Fairlawn, NJ). The elution buffer consisted of a 50:50 (v/v) solution of acetonitrile and 25% phosphoric acid having a final pH of 3.5.

Salvaged human plasma used for the preparation of controls and standards was obtained from the

University of Texas M.D. Anderson Cancer Center Blood Bank. Drug-free urine, used as above, was collected from healthy volunteers within our laboratories with their informed consent.

2.2. Standard stocks

Karenitecin stock solution was prepared by dissolving 20 mg of the drug in 100 ml acetonitrile to a final concentration of 200 μg/ml and stored in 1 ml aliquots at −70°C prior to further dilution with acetonitrile. The internal standard, camptothecin, was prepared by dissolving 10 mg of the drug in 20 ml DMSO to a final concentration of 500 μg/ml. This solution was diluted further by adding 500 μl (250 μg) to 24.5 ml of DMSO yielding a final concentration of 10 μg/ml. This solution was then stored in 1 ml aliquots at −70°C prior to further dilution to 100 ng/ml using DMSO.

2.3. Sample preparation

Karenitecin was isolated from human plasma and urine using solid-phase extraction. In preparation for extraction, 100 mg C_{18} solid-phase extraction cartridges (AccuBond, J&W Scientific, Folsom, CA) were conditioned first with 1 ml of acetonitrile, followed by 1 ml of water. Ten μl (1 ng) of camptothecin, the internal standard, was then added to 1 ml of the sample, plasma or urine, and vortexed for 30 s. The extraction process was facilitated using a vacuum manifold (Supelco, Bellfonte, PA). After application of the sample to the extraction cartridge, the column was then washed twice with 1 ml of HPLC grade water and once with 1 ml of 25% acetonitrile. Finally, the elution and concentration step was achieved by using 500 μl of the elution buffer, a 50:50 (v/v) acetonitrile and 25% H_3PO_4 (pH 3.5). After mixing, 200 μl of the extracted sample was injected into the column. Samples were kept at room temperature prior to injection. Once extracted, samples were stable for at least 24 h at room temperature.

Prior to the karenitecin total drug (total drug=lactone+carboxylate) extraction, 500 μl of patient sample plasma or urine was diluted with 500 μl of 10% H_3PO_4, pH 4.3, and allow to incubate for at least 30 min. This was done to force the conversion of all the drug present to lactone form. After incubation, the karenitecin total drug samples were processed as described above.

2.4. Extraction efficiency

To determine extraction efficiency for plasma, human plasma samples were spiked with karenitecin to achieve a final concentration of 1.0 and 50 ng/ml then extracted. Peak areas of karenitecin obtained from human plasma samples assayed using this method were compared with peak areas of standards which were prepared in 500 μl elution buffers and injected directly onto the HPLC system. Six samples were analyzed for each concentration and matrix. Extraction efficiency was calculated with the following equation: (peak area extracted sample for *X* ng/ml÷peak area neat sample for *X* ng/ml)×100= Percent extraction efficiency. The same method was used to determine the extraction efficiency for human urine with spiked human urine samples with karenitecin to achieve a final concentration of 1.5 and 50 ng/ml.

2.5. Protein binding experiments

Karenitecin is a lipophilic drug with the potential for some degree of protein binding. To evaluate protein binding standard equilibrium dialysis methods were followed. Briefly, Soerensen phosphate buffer (pH 7.4, 0.067 M KH_2PO_4 and Na_2HPO_4 and NaCl) was prepared from analytical grade reagents. Fresh human drug-free plasma was obtained from a normal volunteer. Regenerated cellulose membranes with 12 000–14 000 MWCO were used in each dialysis macro-well (Spectrum Laboratories, Rancho Dominguez, CA). Five plasma samples were spiked for each of the following karenitecin concentration 100, 250, 500 and 1000 ng/ml. After completing the appropriate time-analysis studies, samples were run for 24 h to reach equilibrium. These experiments were repeated in triplicate.

2.6. HPLC apparatus and chromatographic conditions

The HPLC system consisted of Millennium Chromatography Manager 32 version 3.05.01 for integra-

Table 1
Mobile phase gradient profile for karenitecin HPLC assay

Time (min)	Flow-rate (ml/min)	Acetonitrile (%)	Water (%)
1	1	5	95
2	1	20	80
5	1	30	70
10	1	50	50
15	1	60	40
20	1	70	30
25	1	5	95

tion and management of the Alliance 2690 Separations Module, and Waters 474 fluorescence detector (Waters Corporation, Milford, MA). The fluorescence detector was set at an excitation wavelength of 370 nm and emission was monitored at 490 nm. Separation was achieved at ambient temperature (27°C) using an ODS column with the following characteristics: 4 μm particle size, 3.9×150 mm, 60 Å pore size (Nova-Pak C_{18}, Waters, Milford, MA) in combination with a pre-column of identical functionality.

2.7. Separation

This reverse-phase HPLC assay employed gradient elution. The gradient profile ramped from 5 to 70% acetonitrile over 20 min, at a constant flow rate of 1 ml/min, requiring a total run time of 28 min (Table 1).

2.8. Quantification

To validate this analytical method, salvaged human plasma and urine were spiked with authentic karenitecin standard solutions and extracted as described above. The dynamic linear range employed for instrument calibration was 1–50 ng/ml for plasma and 1.5–50 ng/ml for urine. Three calibration standard curves, completed on 3 consecutive days, were obtained by analyzing 5 plasma standard concentrations (1, 5, 10, 20, and 50 ng/ml) or 5 urine standard concentrations (1.5, 5, 10, 20, and 50 ng/ml), in triplicate. These curves were deemed acceptable if the inter-day variability was less than 15%. The best-fit calibration line was determined for each curve by sum-squared linear regression analysis of the calibration data using Millennium Chromatography Manager 32 version 3.05.01 (Waters Corporation, Milford, MA). The inter-day analysis of accuracy and precision included back calculations of concentration for all standard samples using the slope and intercept for each curve. The peak area responses from all standards were fitted to each of the three curves to estimate the nominal valve for each standard concentration with <15% variability within and across each standard curve (Tables 2 and 3). Intra-day analysis of assay precision and assay reproducibility was completed using concentrations of 2.5 (n=25) and 40 ng/ml (n=10) for both plasma and urine. To determine the assay accuracy reproducibility, the mean and coefficient of variance was calculated for the set of samples. The same coeffi-

Table 2
Precision and accuracy of karenitecin measurement in human plasma

Karenitecin nominal Conc. (ng/ml)	Measured concentration (ng/ml)	% R.S.D. (%)	Accuracy (%)
Intra-day assay[a]			
2.5	2.67±0.17	6.4	93.2
40	41.95±2.97	7.1	95.1
Inter-day assay [b]			
1.0	1.04±0.98	9.4	95.6
10	9.97±0.33	3.4	94.7
50	50.14±0.89	1.7	99.7

% R.S.D.=relative standard deviation
[a] Mean±S.D. (n=10).
[b] Mean±S.D. (n=3).

Table 3
Precision and accuracy of karenitecin measurement in human urine

Karenitecin nominal conc. (ng/ml)	Measured concentration (ng/ml)	% R.S.D. (%)	Accuracy (%)
Intra-day assay[a]			
2.5	2.53±0.03	0.7	97.2
40	35.35±1.06	3.0	88.4
Inter-day assay [b]			
1.5	1.53±0.08	4.9	98.0
10	9.97±0.51	5.5	99.7
50	50.24±1.88	3.7	99.5

% R.S.D. = relative standard deviation
[a] Mean±S.D. ($n=10$).
[b] Mean±S.D. ($n=3$).

cient of variance (<15%) was set as the threshold for acceptance of intra-day reproducibility (Tables 2 and 3). Lower limit of detection, or LLD, was defined as the lowest concentration with a peak area response greater than the acceptable signal to noise ratio of 3:1.

The lower limit of quantification, or LLQ, was the lowest concentration which could reliably and repeatedly be measured with <15% variability.

2.9. Stability study

The stability of karenitecin in human plasma stored at −70°C for up to 6 months was evaluated at concentrations of 1 and 50 ng/ml in plasma and 1.5 and 50 ng/ml in urine. Moreover, the effect of freezing and thawing on the stability of karenitecin was evaluated in both plasma and urine at these same concentrations for three freeze–thaw cycles. For the freeze–thaw studies samples of donor plasma were spiked with karenitecin and frozen at −70°C and analyzed at 72 h, 1 week, and 2 weeks from the day of preparation (Day 1). Peak areas from these samples were then compared to results obtained from identical samples prepared fresh, extracted and analyzed on day 1.

3. Results and discussion

We have developed and validated a HPLC method using fluorescence detection for the determination of karenitecin concentrations in human plasma and urine. Unlike many existing camptothecin analog assays, this method did not require the addition of a buffer to the mobile phase to maintain pH, achieve peak separation, or to minimize peak tailing.

Retention time for karenitecin was 16.2±0.5 min and camptothecin at 8.0±0.2 min for both plasma and urine (Fig. 2). The karenitecin peak was baseline resolved, with the nearest peak at 3.1 min distance. Using normal volunteer plasma and urine from multiple individuals, as well as samples from the 50 patients analyzed to date, no interfering peaks were detected. This included patients that were receiving concomitant medications, co-eluting peaks were not detected.

The linear regression equation for karenitecin plasma concentration was: karenitecin plasma concentration = [(observed peak area response − 1.28×10^4)/1.72×10^5], with a correlation coefficient (r^2) of 0.998. Throughout the concentration dynamic range the coefficient of variation ranged from 1.75 to 9.37%. The karenitecin urine assay procedure was found to be linear over a dynamic range from 1.5 to 50 ng/ml. The linear regression equation for karenitecin urine concentration for was: karenitecin urine concentration = [(observed peak area response + 4.11×10^4)/1.78×10^5], with a correlation coefficient (r^2) of 0.998. Throughout the concentration dynamic range coefficient of variation ranged from 3.75 to 4.95%.

Karenitecin peak areas were proportional over the plasma concentration ranges from 1.0 to 50 ng/ml.

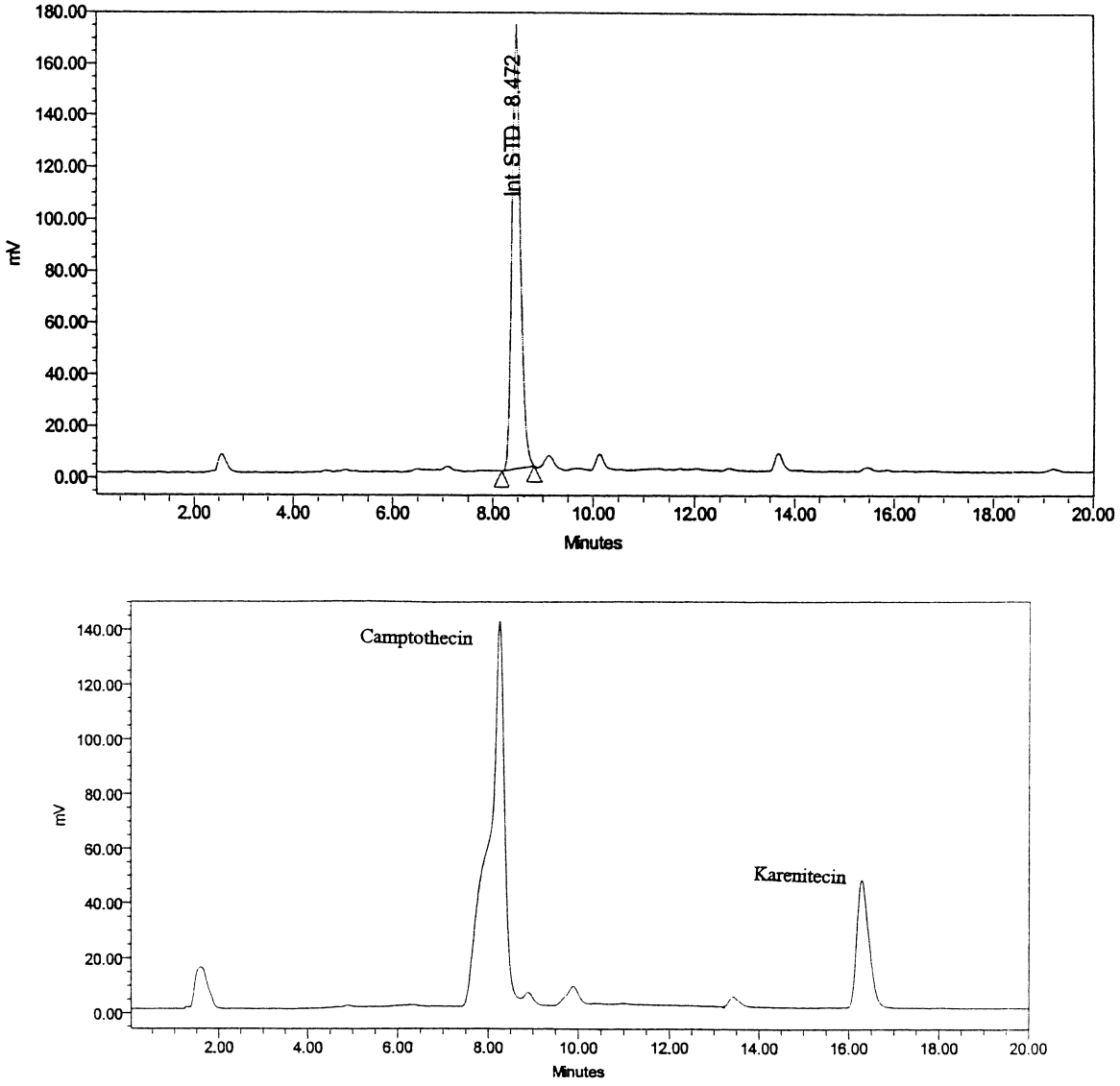

Fig. 2. Chromatograms of (a) salvaged human plasma blank and (b) salvaged human plasma spiked with 5 ng/ml karenitecin obtained with separation achieved by gradient elution, using a water and acetonitrile mobile phase, on a C_{18} analytical column. Karenitecin was detected using fluorescence detection at excitation and emission wavelengths of 370 and 490 nm, respectively. Retention time for karenitecin was 16.2±0.5 and 8.0±0.2 min for camptothecin, the internal standard.

Mean recoveries of karenitecin from plasma at concentrations of 1.0 and 50 ng/ml were 81.9 and 87.82%, respectively. Relative standard deviations (%RSD) of calculated values for the karenitecin plasma calibration standards ranged from 0.43 to 9.87% with relative errors ranging from 0.42 to 12.09%. Similarly, the karenitecin peak areas were proportional over the urine concentration ranges from 1.5 to 50 ng/ml. The mean recovery of karenitecin from urine at concentrations of 1.5 and

50 ng/ml were 90.34 and 78.35%, respectively. Relative standard deviations (%RSD) of calculated values for the karenitecin urine calibration standards ranged from 0.81 to 6.49% with relative errors ranging from 1.18 to 11.82%.

Assay precision, based on an extracted karenitecin standard plasma sample of 2.5 ng/ml, was ±4.46% with a mean accuracy of 92.4%. For extracted karenitecin standard urine samples of 2.5 ng/ml assay precision was ±2.35% with a mean accuracy of 99.5%. The lower limit of detection (LLD) for karenitecin, defined as 3× the detector noise, was 0.5 and 1.0 ng/ml in plasma and urine, respectively. The lower limit of quantification (LLQ) for karenitecin, defined as 4× detector noise with <15% variability for repeated measures, was 1 ng/ml for an extracted 1 ml aliquot of plasma and 1.5 ng/ml for an extracted 1 ml aliquot of urine.

Results of the stability study indicate that when frozen at −70°C, karenitecin is stable in human plasma for up to 3 months and in human urine for up to 1 month. Likewise the freeze–thaw experiments demonstrated that karenitecin was stable in both matrices for up to 3 freeze–thaw cycles (Table 4). In each instance the overall reduction in karenitecin concentration was smaller than the co-efficient of variation for the assay (<15%). There was some variability (<15%) in the rate of degradation between the high and low concentrations.

Protein binding studies confirmed this lipophilic compound, karenitecin is highly protein bound. The mean percent protein bound drug is 99.1±0.27%. The protein binding did not appear to be concentration dependent with the mean percent protein bound drug ranging from 98.8 to 99.34% for the five karenitecin concentrations evaluated within the range of 100–1000 ng/ml.

In conclusion, we have reported a highly sensitive, liquid chromatographic method for quantifying karenitecin in human plasma and urine. No human plasma or urine components were observed at the retention time of karenitecin. This assay method has been validated in both human plasma and urine and will be useful for the analysis of samples from phase I and II pharmacokinetic studies of karenitecin.

Currently, phase I and II trials for karenitecin are ongoing. Patients have achieved peak karenitecin concentrations ranging from 6.3 to 49.1 ng/ml over the dosage range of 150–2400 μg/m^2/d given as a 60-min infusion. Mean peak concentrations at this dose level are well within the dynamic range of this assay. The LLQ for both matrices allows for the determination of karenitecin for >24 h after the administration of these dosages.

Table 4
Stability of karenitecin in human plasma and urine

Storage condition	Matrix/nominal concentration		Mean % of nominal concentration (%)	RSD (%)	CV (%)
Continuous storage					
−70°C ×90 days (n=3)	Plasma	1 ng/ml	94.0	7	8.1
		50 ng/ml	89.6	2.94	3.6
−70°C ×30 days (n=3)	Urine	1.5 ng/ml	105.4	2.0	2.8
		50 ng/ml	83.6	2.26	2.8
Freeze/Thaw*					
−70°C ×3 cycles (n=3)	Plasma	1 ng/ml	102.7	4.77	2.3
		50 ng/ml	90.6	4.22	1.3
−70°C ×3 cycles (n=3)	Urine	1.5 ng/ml	101.6	3.67	6.4
		50 ng/ml	90.3	3.17	0.9

* Freeze/Thaw cycles consisted of thawing, analysis, and re-freezing at 72 h, 1 week, and 2 weeks from the initial freezing.

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