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A Rapid Ultra HPLC-MS/MS Method for the Quantitation and Pharmacokinetic Analysis of 3-Deazaneplanocin A in Mice

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Abstract

3-Deazaneplanocin A (DZNep) has been shown to have anti-cancer activity in numerous cancer types and its continued preclinical, and eventual clinical, drug development will require rapid and sensitive bioanalytical methods in order to quantitate this drug for pharmacokinetic analyses. The ultra HPLC with positive electrospray tandem mass spectrometric (LC-MS/MS) detection affords the most sensitive (limit of quantitation 5 ng/mL) and rapid (3 min run time) bioanalytical method to date for DZNep. Due to the polar nature of this drug and the internal standard (tubercidin), a hydrophilic-interaction column (HILIC) was used. The method was accurate, with less than 10% deviation from nominal values, as well as precise, where both within-day and between-day precisions were less than 15%. A liquid-liquid extraction procedure was able to recover ~90% of drug from a small volume (50 µL) of mouse plasma. This method was successfully applied to a pharmacokinetic study in mice intravenously injected with DZNep.

Keywords

3-Deazaneplanocin; Ultra HPLC-MS/MS; Pharmacokinetics

1. Introduction

Epigenetic chromatin remodeling, such as DNA methylation and histone modifications, is a highly regulated pathway of gene expression and silencing that maintain normal genomic

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order. Alterations in these pathways, such as histone methylation and deacetylation, can result in tumorigenesis [1–4]. Gene silencing pathways that are known to be altered in cancer are DNA hypermethylation [5] and histone methylation via Polycomb repressor complex 2 (PRC2) [6, 7]. PRC2 is mainly responsible for trimethylation of lysine 27 on histone 3 (H3K27m3), which represses several key tumor suppressor pathways [8], and consists of the catalytic enhancer of zeste homolog 2 (EZH2) subunit, as well as the suppressor of zeste 12 (SUZ12) and embryonic ectoderm development (EED) proteins. While EZH2 is expressed in very low levels in a variety of normal tissues [9], it is overexpressed in a wide range of cancers, including breast [10, 11], gastric [12], cutaneous melanoma [10], prostate [10, 13], renal cell carcinoma [14], pleural mesothelioma [15], and uterine [10]. Depletion of EZH2 caused increased apoptosis and senescence, while proliferation of EZH2 decreased apoptosis in cancer cells [16–18]. Such evidence suggesting inhibition of EZH2 and similar pathways can ultimately reactivate tumor suppressor genes makes them a desired target for anti-cancer therapeutics.

3-Deazaneplanocin A (DZNep), a cyclopentanyl analog of 3-deazaadenosine, is a global histone methylation inhibitor that is known to inhibit histone methyltransferase activity indirectly through EZH2 as well as inhibiting other methyltransferases [19]. Furthermore, DZNep inhibited cell proliferation and tumor growth in mesothelioma [15] in addition to other cancers [20–22]. Due to the potential therapeutic benefit offered by DZNep, there is a need for further preclinical evaluation and bioanalytical assays to quantify DZNep in biological fluids for pharmacokinetic studies. To date, only a few HPLC-based bioanalytical methods have been developed for DZNep [23–25], each with considerably long run times (by modern standards) ranging from 12 to 42 min, and a lower limit of quantification (LLOQ) of 20 ng/mL for comparable plasma assays [25]. Liposomal DZ-Nep was measured using a similar assay [25], with a LLOQ of 500 ng/mL [24].

The purpose of the present work was to validate a more rapid and sensitive ultra HPLC-MS/MS method for the quantification of DZNep in murine plasma (50 μ L) for application towards preclinical pharmacokinetic studies. This method is ideally suited for such experiments in mice, which typically provide small plasma volumes. To date, the validated method described here achieved the fastest run time (3 min), while demonstrating the greatest sensitivity, with a LLOQ of 5 ng/mL.

2. Experimental

2.1. Materials

3-Deazaneplanocin (DZNep) was supplied by the Laboratory of Medicinal Chemistry of the National Cancer Institute (Bethesda, MD). Tubercidin (internal standard) and ammonium acetate was purchased from Sigma-Aldrich (St. Louis, MO). Methanol, acetonitrile, ethyl acetate, dimethyl sulfoxide (DMSO) and isopropanol were purchased from Fisher Scientific (Pittsburgh, PA). Drug-free Balb-C mouse plasma with sodium heparin was purchased from Innovative Research (Novi, MI). All water used was generated with a Hydro-Reverse Osmosis system (Durham, NC, USA) connected to a Milli-Q UV Plus purifying system (Billerica, MA, USA).

2.2. Preparation of Stock Solutions

Stock solutions were prepared individually by dissolving DZNep and tubercidin in DMSO to achieve concentrations of 1 mg/mL, which were stored at -80°C . Serial dilutions containing DZNep were prepared in acetonitrile and stored at -80°C for the preparation of calibration and quality control (QC) samples. The chemical structures of DZNep and tubercidin are pictured in Figure 1.

For each analytical run, calibration standards in drug-free heparinized mouse plasma were freshly prepared in duplicate at concentrations of 5, 10, 50, 100, 500, 1,000, and 2,000 ng/mL. Quality control (QC) samples were prepared in batch at concentrations of 9, 900, and 1,800 ng/mL, and a 10-fold dilution QC up to 10,000 ng/mL, through addition of plasma to the required amount of working stock, then aliquotted and stored at -80°C .

2.3. Sample Preparation

Frozen standards and samples were thawed on wet ice before homogenization by vortex-mixing. DZNep was extracted from 50 μL of mouse plasma by the addition of 200 μL of 0.1 M ammonium acetate, pH 9, followed by 500 μL of 500 ng/mL tubercidin in (15/85, v/v) isopropanol/ethyl acetate and then an additional 1.5 mL of clean (15/85, v/v) isopropanol/ethyl acetate. The samples were vortexed, centrifuged, and the organic layer evaporated to dryness before reconstitution with 100 μL of acetonitrile (ACN).

2.4. Instrument Conditions

The samples were chromatographically separated with a Waters Acquity UPLCTM system (Waters Corporation, Milford, MA, USA), which included a binary pump, a refrigerated autosampler, and a temperature-controlled column compartment. The injection volume was 10 μL , with the autosampler maintained at 4°C , and the column compartment at 40°C . Because both compounds are polar bases, chromatographic separation was achieved on a Waters Acquity UPLCTM BEH HILIC (hydrophilic interaction chromatography) column (100×2.1 mm, internal diameter) and HILIC guard column (5×2.1 mm) packed with 1.7- μm packing material. The mobile phase consisted of A: (95/5, v/v) 10 mM ammonium acetate, pH 5/methanol, and B: (95/5, v/v) 0.2% formic acid in ACN/10 mM ammonium acetate, pH 5. The percent of mobile phase A was held at 9% for the first 0.5 min, increased linearly to 40% over the next 0.5 min before holding at 40% for another min, then finally back to 9% to equilibrate the column prior to the next injection. The run time was 3 min and the flow rate was 0.5 mL/min. The LC column eluent was directed into an AB Sciex QTrap 5500 mass spectrometer (AB Sciex, Foster City, CA, USA). The mass spectrometer was set to monitor DZNep (m/z 263.1 $\rightarrow$ 135.1) and tubercidin (m/z 267.1 $\rightarrow$ 135.1) using multiple reaction monitoring (MRM) in the positive ion mode. The mass transitions were previously described [25]. Universal mass spectrometric settings included ion spray voltage of 5500 V, source temperature of 500°C , GS1 and GS2 at 45 psi and 30 psi, respectively, entrance potential of 12 V, collision exit potential of 13 V, declustering potential of 135 V, and dwell times of 200 msec for each mass transition. MRM peak integrations and data analyses were performed using MultiQuant 4.0 (Analyst[®] v1.2, AB Sciex, Foster City, CA).

2.5. Validation

2.5.1. Linearity, Accuracy and Precision—Calibration curves for DZNep were constructed by least-squares linear regression analysis of a seven-point calibration curve by plotting peak area of the analyte versus the peak area of the internal standard (tubercidin), using $1/\chi^2$ as a weighting factor. Calibrator response functions and choice of regression analysis were investigated by calculating correlation coefficients (r) and the percent deviation (% DEV) for all calibrators.

Accuracy and precision were evaluated by quantifying DZNep at four different concentrations of QC samples in five replicates analyzed over four different days. Each run consisted of blank plasma extracts, internal standard only extracts, calibration standards in duplicate, and QC samples in replicates of five. Accuracy (% DEV) was defined as the percent difference between the mean observed concentration and the nominal concentration. The repeatability of the assay was determined by the within-run precision (WRP) and between-run (BRP), as calculated below.

$$WRP=100 \times \left[(MS_{WT})^{0.5} / GM \right]$$

$$BRP=100 \times \left[\left(\{MS_{BET} - MS_{WT}\} / n \right)^{0.5} / GM \right]$$

GM represents the grand mean over the four days, *MS_{wt}* represents the within-group mean squared, *MS_{bet}* represents the between-group mean squared, and *n* represents the number of repetitions (*n*=20, with QCs analyzed in quintuplet over four days). FDA guidelines for bioanalytical accuracy and precision were followed, with $\pm 15\%$ variability allowed [26].

2.5.2. Stability—Stability tests were performed to verify the stability of DZNep during freeze/thaw cycles. Samples were assayed at three calibrator concentrations (9, 900, and 1,800 ng/mL). The samples were subjected to four freeze/thaw cycles at -80°C , with each freeze cycle lasting at least 12 hr. The concentration of the drugs after each storage period was compared to the concentration of freshly prepared samples in the same analytical run.

The plasma stability of DZNep was assessed at three concentrations by comparing QCs in plasma that remained at room temperature on the bench for 24 hrs to freshly thawed and extracted QCs of the same batch. The post-preparative stability was assessed by preparing samples at three concentrations and measuring after a fresh extraction and then again 24 hours later while at 4°C in the HPLC autosampler.

2.5.3. Matrix Effects and Extraction Efficiency—The extraction efficiency (recovery) of DZNep from mouse plasma following a liquid-liquid extraction was assessed by comparing analyte peak areas before (set B) and after (set A) the addition of the liquid extraction solution. Recovery was performed at both a low (10 ng/mL; *n*=3) and high (1,000 ng/mL; *n*=3) concentration and was calculated by dividing analyte peak areas from set A by set B ($A/B \times 100\%$).

Matrix effects from plasma were assessed through direct comparison of samples spiked in plasma after extraction (set A) to samples spiked in the reconstitution solution (acetonitrile; set C) at both a low (10 ng/mL; *n*=3) and a high (1,000 ng/mL; *n*=3) concentration. Matrix effects were calculated by dividing peak areas from set A by set C ($A/C \times 100\%$). The purpose of the comparison of peak area between neat solution and plasma following extraction was to eliminate recovery as a source of systematic error.

2.6. Preclinical Pharmacokinetic Application

Mice were injected intravenously with 2.5 mg/kg of DZNep and were sacrificed at the following time points for pharmacokinetic analysis: pre-dose, 10, 15, 25, 45, 60, 90, and 180 min post-dose. The plasma concentration vs time data was subsequently analyzed using a two-compartment model (WinNonlin v5, Pharsight Corp, Mountain View, CA).

3. Results and Discussion

Figure 2 depicts typical chromatograms resulting from the uHPLC-MS/MS analysis of extracts of 50 μL mouse plasma from a: (A) blank plasma extract, (B) IS-only extract, and (C) the lower limit of quantitation (LLOQ; 5 ng/mL). The DZNep and IS peaks were chromatographically separated under the optimized conditions, with retention times for IS (bottom), and DZNep (top) of 0.95 and 1.58 min, respectively. Peak shape, resolution, and

signal to noise were acceptable to meet FDA criteria. No carryover was observed for either analyte or IS by running a blank sample following the upper limit of quantification (ULOQ; 2,000 ng/mL) and not detecting any analyte or IS peaks at their respective retention times.

3.1 Linearity

The DZNep calibration curve spanned the plasma concentration range of 5 – 2,000 ng/mL, and was back-calculated from the peak area ratio of DZNep:IS and the y-intercept. The deviation for all concentrations from the nominal concentrations fell within acceptable limits, between –4.40% and 4.07%, whereas the precision (measured by percent coefficient of variation or %CV) ranged from 5.24 to 10.63% (Table 1). The mean ($\pm$ standard deviation) correlation coefficient obtained on four separate days resulted in a mean $r^2 = 0.9951 \pm 0.0015$ ($n = 4$). The model with the least total bias across the concentration range investigated was obtained using $1/\chi^2$ as the weighting factor.

3.2. Accuracy and Precision

The assay performance data for the determination of independent QC samples of DZNep in mouse plasma are presented in Table 2. The deviation from nominal concentration (accuracy) ranged from –4.42% to 8.13% and within-run and between-run precision was all less than 15%.

3.3. Stability

DZNep short-term plasma stability was not a factor, as none of the three concentration levels had more than a 15% decrease in calculated concentration after 24 hr at room temperature prior to extraction. Long-term plasma stability (>24 hrs) was not assessed. No significant degradation greater than 15% was observed following two freeze/thaw cycles of plasma samples containing DZNep at plasma concentrations of 9, 900, or 1,800 ng/mL. Post-preparative stability demonstrated less than 2% difference between extracted samples analyzed immediately or after 24 hr at 4 °C in the autosampler. Extraction efficiency was 91.81% and 89.17% for the low (1 ng/mL, $n=3$) and high (1,000 ng/mL, $n=3$) concentrations, respectively, which is greater than that of a previous studying using a much more cumbersome and time-consuming solid-phase extraction followed by overnight lyophilization that achieved 70–86% recovery from rat plasma and other rat matrices [25]. Significant matrix effects were observed for the low (84%) and high (87%) concentrations.

3.4 Preclinical Application

Figure 3 depicts the log-transformed plasma concentration vs. time curve, where DZNep exhibited biphasic elimination. A two-compartment pharmacokinetic (PK) model was applied to the data in order to obtain PK parameters, including a clearance of 0.09 L/min/kg and a terminal half-life of 0.9 hr. The clearance and half-life values are similar to a previous study, where 1 mg/kg DZNep was administered intravenously to rats [24].

Conclusions

3-Deazaneplanoncin A was accurately, precisely, and rapidly quantified in mouse plasma with the use of uHPLC-MS/MS, which allowed for sharp chromatographic peaks with sufficient separation using a short run time of 3 min. The ultra HPLC provided the necessary performance to make this assay the fastest analytical method for DZNep to date. The method provided sufficient sensitivity for pharmacokinetic studies in mice (5 ng/mL), where DZNep plasma concentrations were still greater than the LLOQ at the last time point drawn, 3 hours post intravenous dose. The small starting volume of mouse plasma (50 μ L) is especially

important because of the low blood volume available in a single mouse for routine pharmacokinetic sampling.

This uHPLC-MS/MS with a simple liquid extraction can ultimately be applied to other preclinical and clinical biosamples (plasma, urine, tissue, etc) for future pharmacokinetic studies.

Abbreviations

DZNep	3-Deazaplanocin A
uHPLC	Ultra-high performance liquid chromatography
MS	mass spectrometry
MS/MS	tandem mass spectrometry
QC	Quality control
LLOQ	Lower limit of quantification
IS	internal standard

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***Highlights**

We developed and validated a novel uHPLC-MS/MS assay for the quantification of 3-deazaneplanocin A (DZNep).>This method has a faster run time and is more sensitive than previous methods.>This method is accurate, precise, and a simple liquid extraction procedure recovers 90% of drug from mouse plasma.>Successfully applied to preclinical pharmacokinetic study in mice.

3-Deazaneplanocin A (DZ-Nep)

Tubercidin (Internal Standard)

Figure 1.
Structure of DZNep and Tubercidin

Figure 2. Chromatogram of A) Blank Plasma Extract, B) IS-only Extract, and C) Lower Limit of Quantification.

The top panel represents a blank plasma extract, middle panel represents the IS-only extract, and the bottom panel represents the lower limit of quantification (LLOQ) of 5 ng/mL. There are two separate LC-MS/MS chromatographic tracings within each panel resulting from two simultaneous multiple reaction monitoring (MRM) transitions. The bottom pane within each panel represents the internal standard tubercidin (m/z 267.1 $\rightarrow$ 135.1) and the top pane represents DZNep (m/z 263.1 $\rightarrow$ 135.1).

Figure 3.
Preclinical Concentration-Time Curves
DZNep was administered intravenously to mice at a dose of 2.5 mg/kg. A two-compartmental model was used to obtain pharmacokinetic parameters.

Table 1

Calibration Linearity

Nominal (ng/ml)	GM (ng/ml)	S.D. (ng/ml)	DEV (%)	CV (%)	n
5	4.95	0.37	−0.92	7.37	8
10	10.11	0.71	1.07	7.04	8
50	51.58	2.70	3.16	5.24	8
100	100.45	9.75	0.45	9.70	8
500	520.33	42.52	4.07	8.17	8
1,000	955.97	84.89	−4.40	8.88	8
2,000	1930.81	205.27	−3.46	10.63	8

Abbreviations: GM, grand mean; S.D., standard deviation; DEV (%), relative deviation from nominal value; CV (%), coefficient of variation; *n*, number of replicate observations within each validation run, i.e. two samples at each concentration were run on four separate occasions, for a total (*n*) of eight samples at each concentration.

Table 2

Accuracy and Precision

Nominal (ng/ml)	GM (ng/ml)	S.D. (ng/ml)	DEV (%)	WRP (%)	BRP (%)	n
9	8.90	0.99	-1.12	9.61	6.36	20
900	957.12	133.99	6.35	13.58	3.82	20
1,800	1720.41	224.32	-4.42	11.51	6.88	20
10,000	10,813.10	968.36	8.13	8.67	4.42	20

Abbreviations: GM, grand mean; S.D., standard deviation; DEV (%) relative deviation from nominal value; WRP, within-run precision; BRP, between-run precision; *n*, number of replicate observations within each validation run.