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Article

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Development and Validation of LC–MS-MS Assay for the Determination of the Emerging Alkylating Agent Laromustine and Its Active Metabolite in Human Plasma

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Abstract

The objective of this study was to validate a method for the determination of laromustine (VNP40101M) and short-lived its active metabolite (VNP4090CE) that has a half-life in human blood of <90 s in human plasma by liquid chromatography (LC) with tandem mass spectrometric (MS/MS) detection. We overcome the stability dilemma by acidified the human plasma with citric acid. Laromustine "breaks" down on the source of mass spectrometry to give *m/z* 249 which is the same *m/z* for VNP4090CE. Because VNP4090CE and laromustine elute at approximate retention time of 1.93 and 2.94 min, respectively, we were able to quantify both of them in one method. VNP40101M, VNP4090CE and the internal standards were extracted from human plasma by liquid–liquid extraction into ethyl ether. The ethyl ether layer was evaporated, reconstituted and analyzed using LC with MS/MS detection. Validation parameters such as selectivity, limit of quantitation, linearity, precision, accuracy, recovery, autosampler viability, freeze–thaw cycles and compounds stability are evaluated for this method. Results were calculated using peak area ratios, and calibration curves were generated using a weighted ($1/x^2$) linear least-squares regression. Calibration curves for VNP40101M and VNP4090CE in human plasma ranged from 1.00 to 1,000 ng/mL. In this study, both intra- and inter-assay results demonstrated a relative standard deviation for calibration standards (inter-assay) and quality control samples (intra- and inter-assay) to be ≤15.0%. In this method, there is ~1.79% isotopic interference of VNP40101M to VNP40101M-IS, and ~3.76% isotopic interference of VNP4090CE to VNP4090CE-IS. It was concluded that there was no significant carryover.

Introduction

Alkylating agents are capable of introducing an alkyl group into nucleophilic sites on DNA or RNA through covalent bond (1). There are several groups of alkylating agents such as nitrogen mustards (e.g., mechlorethamine, cyclophosphamide, ifosfamide, chlorambucil, bendamustine and melphalan); ethylenimines (e.g., thio-tepa); nitrosoureas (e.g., carmustine, lomustine, streptozocin); triazines (e.g., dacarbazine, temozolomide); methylhyderazines (e.g., procarbazine) and alkyl sulfonates (e.g., busulfan). Originally, alkylating agents were best known for their use as mustard gas and related chemical weapons in World War I, due

to their toxicity. These agents are thought to react with the N7 position of guanine or any nitrogen base in each of the double strands of DNA, resulting in damage to the DNA (1). They are harmful to normal cells, especially cells that divide frequently, such as those in the gastrointestinal tract, bone marrow, testicles and ovaries, which can cause loss of fertility. As early as the 1940s, it was discovered that they could be used as a part of chemotherapy in different types of cancers (2–4). One early example is busulfan, a cancer drug in use since 1959; in 1999, it was approved by the US Food and Drug Administration (FDA) for treatment of chronic myeloid leukemia (CML). Busulfan was the main chemotherapeutic for the treatment of CML until it was displaced by imatinib, but it is still in use because of its low cost. Alkylating agents are commonly used to treat many different cancers, including leukemia, lymphoma, Hodgkin's disease, multiple myeloma and sarcoma, as well as cancers of the lung, breast and ovary. The risk with these drugs is that, at higher doses, they can cause long-term damage to the bone marrow.

The sulfonylhydrazines are a new family of potent alkylating agents, from which laromustine (1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2-[(2-methylamino)carbonyl] hydrazine) hydrazine, also known as VNP40101M, was selected for clinical development (5–13). The chemical structures of laromustine and its active metabolite VNP4090CE (half-life in blood <90 s) are shown in Figure 1a and the pathway of activation of laromustine in Figure 1b. To perform our assessment of the mass spectral rearrangement of laromustine, we used mass spectrometry with an electrospray ionization interface. Mass spectrometry has long been a major component in the analysis of structure information in complex biological matrices (14–22). Time-of-flight MS, FTICR-MS and Oribtrap mass spectrometers are among the most effective for this work. With recent advances in FTICR-MS, it has become routine to generate mass spectra with ppm mass accuracy and exceptional resolving power, two metrics which are critical to determining fragmentation ions for metabolite identification for drug metabolism studies. The online H-D method was developed for use with small molecules where online exchange on column without any further sample preparation is employed for metabolite identification and characterization. H-D has become common practice for online analysis of metabolite identification of complex matrices; when combined with modern mass spectrometry, it has proven ideal for the task of structure confirmation (23–25).

Recently, we published the results of several *in vitro* and *in vivo* studies that were designed to examine the biotransformation and rearrangement of laromustine. High mass accuracy and ultrahigh-resolution measurements, H-D, stable-isotope-labeled analog (^{13}C-labeled laromustine), NMR and detailed analyses of the LC–MS^n experiments were used to assist with the assignments of these fragments and possible mechanistic rearrangement. The results showed that laromustine undergoes rearrangement, dehalogenation and hydrolysis at physiological pH to form active moieties. Laromustine produces several reactive metabolites which were trapped by glutathione, "*N*-acetylcysteine" and cysteine in the *in vitro* systems (18, 23).

Herein, we report for the first time the development and validation of LC–MS-MS assay for the determination of the emerging alkylating agent laromustine and its active metabolite in human plasma. This method was succesful used to study Phase II clinical trials in patients with AML, patients received laromustine 600 mg/m^2 IV. The results showed that there was no significant carryover. The lower limit of quantitation (LLOQ) for VNP40101M and VNP4090CE in human plasma was set at 1.00 ng/mL. VNP40101M and VNP4090CE in human plasma were stable for 365 days when stored in a freezer set to maintain −10 to −30°C, and for 371 days when stored in a freezer set to maintain −60 to −80°C before analysis.

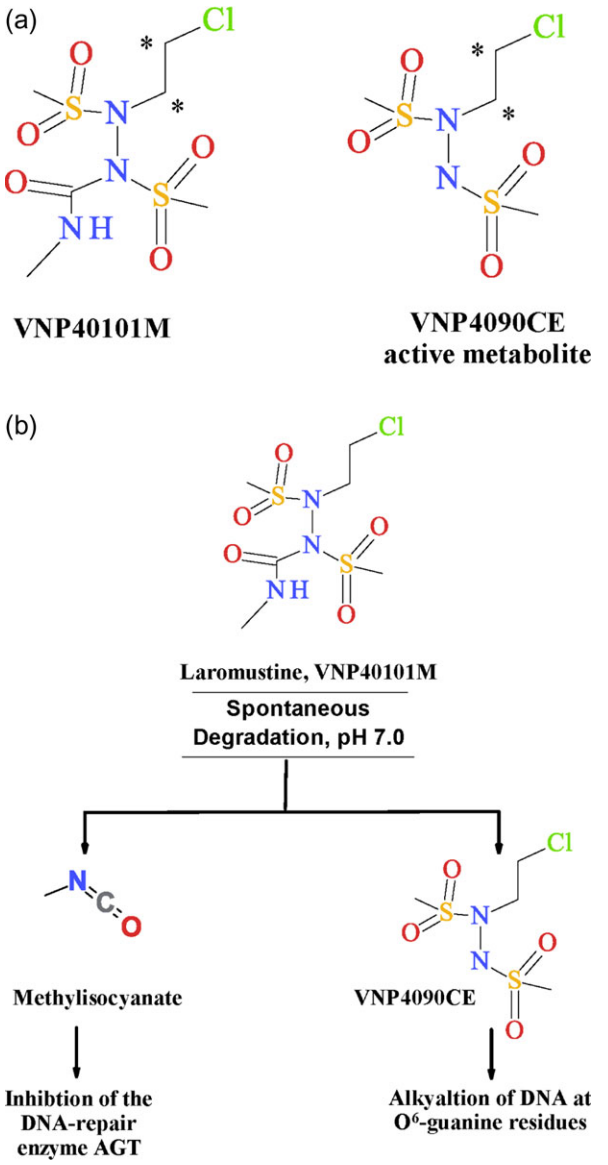

Figure 1. (a) Chemical structures of laromustine and VNP4090CE and (b) the pathway of activation of laromustine.

Materials and Methods

Reference standards and matrix

VNP40101M, Lot No. 04-12-0085a, the purity of VNP40101M was 99.7%. Upon receipt, the compound was stored in a freezer set to maintain −10 to −30°C, desiccated and protected from light. VNP4090CE (also known as 90CE), Lot No. KL367-97, the purity of VNP4090CE was assigned by Covance as 100%. Upon receipt, the compound was stored in a freezer set to maintain −10 to −30°C, desiccated and protected from light. VNP40101M-IS (also known as 2-chloroethyl-^{13}C2), Lot No. PR-16480, the purity of

VNP40101M-IS was ≥98%. Upon receipt, the compound was stored in a freezer set to maintain −10 to −30°C, desiccated and protected from light. "VNP4090CE-IS (also known as 90CE-IS), Lot No. PR-16479 purity was ≥98%". Upon receipt, the compound was stored in a freezer set to maintain −10 to −30°C, desiccated and protected from light. Blank human plasma with sodium heparin was supplied by Biochemed and stored in a freezer set to maintain −10 to −30°C, or below. Blank human plasma was treated with citric acid prior to use (plasma: 2.0 M citric acid = 40:1, *v/v*).

Laromustine high-quality control sample

A quality control (QC) pool was prepared at the high-quality control (HQC) level containing parent analyte (VNP40101M) only. This QC pool was tested to monitor for the breakdown of parent analyte laromustine in plasma into its active metabolite (VNP4090CE). This pool is referred to as the parent (laromustine) high-quality control (PHQC) sample.

Study design

Method validation in this work consisted of demonstrating or establishing method selectivity, limit of quantitation (LOQ), linearity, precision, accuracy, sample carryover, recovery, autosampler viability, freeze–thaw cycle and compounds stability. This method was validated, and the mean intra- and inter-day accuracies and precisions were in accordance with FDA guidelines (26). The validation was designed to consist of at least three precision and accuracy batches, each containing a minimum of the following:

- A calibration standard curve consisting of single replicates of calibration standards at concentrations of 1.00–1,000 ng/mL of VNP40101M and VNP4090CE in human plasma.
- At least one reagent blank, one blank human plasma and one control zero (CTL-0) [blank human plasma fortified with internal standard (ISTD) only].
- Six replicates of QC samples at each of the low QC [LQC (3.00 ng/mL)], medium QC [MQC (150 ng/mL)], high QC [HQC (750 ng/mL)] and PHQC (750 ng/mL) samples.

Selectivity, recovery and frozen matrix stability evaluation batches were designed to include a minimum of the following:

- A calibration standard curve consisting of single replicates of calibration standards.
- At least one reagent blank, one blank human plasma and one CTL-0.
- Two replicates of QC samples at each of the LQC, MQC and HQC concentrations.

The validation was designed to investigate the following:

- Selectivity: at least six samples of blank matrix from different lots.
- Selectivity: at least six samples of blank matrix from these same lots, spiked before extraction near the median concentration of the calibration standards, including ISTD.
- Selectivity: to evaluate matrix effect, extracts of blank matrix from the same lots were spiked post-extraction near the median concentration of the calibration standards, including ISTD. In addition, three replicates of a pure solution containing VNP40101M and VNP4090CE and ISTD at the same concentration as the spiked extract were injected. In addition, one replicate of a pure solution containing VNP40101M, VNP4090CE and ISTD to monitor for potential interference were injected.
- Carryover: an extract of blank matrix run immediately following either of the two highest calibration standards or a HQC sample; at least one positioned early in the batch and at least one toward the end of the batch.
- Precision and accuracy (inter-assay): in three batches, a calibration curve consisting of at least eight calibration levels.
- Precision and accuracy (inter-assay): in three batches, six replicates of LQC, MQC, HQC and PHQC samples.
- Precision and accuracy (intra-assay): in at least one batch, six replicates of LQC, MQC, HQC and PHQC samples.
- Sensitivity: six replicates of LLOQ samples.
- Sample dilution analysis: Six replicates of dilution QC [DQC (7,500 ng/mL)] samples, diluted 10-fold into the calibration range.
- Recovery: three replicates of recovery samples at concentrations corresponding to the LQC, MQC and HQC samples, compared to six replicates of the extracted LQC, MQC and HQC samples.
- Short-term matrix stability: six replicates of LQC, HQC and PHQC samples subjected to at least three freeze/thaw cycles, with QC samples stored at −10 to −30°C and with QC samples stored at −60 to −80°C.
- Short-term matrix stability: six replicates of LQC, HQC, PHQC samples stored at room temperature for at least 4 h before processing.
- Short-term matrix stability: six replicates of LQC, HQC, PHQC samples stored on wet ice for at least 4 h before processing.
- Frozen matrix stability: six replicates of LQC, MQC, HQC and PHQC samples stored in a freezer set to maintain −10 to −30°C and −60 to −80°C for at least for at least 1 year.
- Processed-sample viability: an extracted calibration curve, and six replicates of LQC, MQC, HQC and PHQC samples, processed and stored under conditions defined in the method (refrigerated) for at least 48 h.
- Batch size determination: additional extracted samples were added to at least one batch, such that the batch size was consistent with any prospective study sample batch.

Frozen matrix stability

To evaluate frozen matrix stability, LQC, MQC, HQC and PHQC samples were stored in freezers set to maintain −10 to −30°C and −60 to −80°C. Six replicates of LQC, MQC, HQC and PHQC samples were extracted after storage for 365 days in a freezer set to maintain −10 to −30°C, and after storage for 371 days in a freezer set to maintain −60 to −80°C.

Chromatography and system evaluation

The peak shape and resolution of the peaks of interest must be adequate and consistent throughout the validation. A system evaluation test was performed in the validation. As a minimum, this test evaluated the retention time window and the system response.

Principles of the method

This method is applicable to the analysis of VNP40101M (laromustine) and VNP4090CE in acidified human plasma treated with sodium heparin anticoagulant. VNP40101M, VNP4090CE and the

ISTD, VNP40101M-IS and VNP4090CE-IS, are extracted from human plasma by liquid–liquid extraction into ethyl ether. The ethyl ether layer is evaporated, reconstituted and analyzed using liquid chromatography (LC) with tandem mass spectrometric detection (MS/MS). The standard curve range is from 1.00 to 1,000 ng/mL for VNP40101M and VNP4090CE, using a plasma sample volume of 0.100 mL.

Sample Analysis

Chromatographic conditions

Analytical column Acquity UPLC BEH C18, 50 × 2.1 mm, 1.7 μm was used and Waters Pre-Column Filter, Acquity UPLC Column In-Line Filter, 0.2 μm was used. The column temperature was 35°C. Mobile phase was consistent of 5 mM ammonium acetate in water (A) and acetonitrile:methanol (50:50, *v/v*) (B).

Gradient program			
Time (min)	Flow (mL/min)	A %	B %
Initial	0.5	99	1
2.5	0.5	85	15
3.0	0.5	85	15
3.5	0.5	99	1
4.0	0.5	99	1

Flow rate	0.650 mL/min
Back pressure	500 bar (typical),
Pressure limits	Low: 0 bar; high: 1,000 bar
Sample tray temp	5°C
Injection volume	10–20 μL (typical)
Acquisition time	Approximately 4.0 min
Cycle time	Approximately 4.5 min (injection start to next injection start)

Injector wash solutions: seal wash solution: acetonitrile:water (10:90, *v/v*), weak wash solution: methanol:water:formic acid (50:50:1, *v/v/v*), strong wash solution: 0.5% ammonium hydroxide in water, rinse volume: weak wash solution: 500 μL strong wash solution: 500 μL

Mass spectrometer parameters

Period 1

Period time (min)	2.50	
Mass spectrometer	Sciex API 5000	
Ionization	Negative ion electrospray (ESI^-)	
Mode	MRM	
Ionspray voltage	−4,500 V TurboIon Spray	
Temp	450°C	
Curtain gas type	Nitrogen	Setting: 30
CAD gas type	Nitrogen	Setting: 2
Nebulizing gas (gas1) type	Nitrogen	Setting: 50
Auxiliary gas (gas 2) type	Nitrogen	Setting: 10
Needle position	$Y = 5$ mm, $X = 5$ mm	

Summary of the MRM parameters for VNP4090CE:

Compound ID	Compound name	Transition monitored	Dwell time (ms)	Collision energy (eV)	Approximate retention time (min)
Analyte 2	VNP4090CE	249–115	100	−10	1.93
ISTD2	VNP4090CE-IS	**251**–117	100	−10	1.93

Period 2

Period time (min)	1.50	
Mass spectrometer	Sciex API 5000	
Ionization	Negative ion electrospray (ESI^-)	
Mode	MRM	
Ionspray voltage	−4,500 V TurboIon Spray	
Temp	450°C	
Curtain gas type	Nitrogen	Setting: 30
CAD gas type	Nitrogen	Setting: 2
Nebulizing gas (gas1) type	Nitrogen	Setting: 50
Auxiliary gas (gas 2) type	Nitrogen	Setting: 10
Needle position	$Y = 5$ mm, $X = 5$ mm	

Summary of the MRM parameters for VNP40101M:

Compound ID	Compound name	Transition monitored	Dwell time (ms)	Collision energy (eV)	Approximate retention time (min)
Analyte 1	VNP40101M	249–115	100	−10	2.94
ISTD1	VNP40101M-IS	**251**–117	100	−10	2.94

Calculations

Results were calculated using peak area ratios. Calibration curves for VNP40101M and VNP4090CE in human plasma ranged from 1.00 to 1,000 ng/mL and were generated using a weighted ($1/x^2$) linear least-squares regression. The concentration of VNP40101M and VNP4090CE in the QC samples, as measured by their peak area ratios, was determined from the calibration curves.

Results

Method development for laromustine and its active metabolite in human plasma

LC method with mass spectrometric detection was developed for simultaneous analysis of laromustine and its active metabolite (VNP4090CE) levels in human plasma. Mobile phase was consistent of 5 mM ammonium acetate in water (A) and acetonitrile:methanol (50:50, *v/v*) (B), deemed most appropriate and gave a better peak shape, spectral response and sensitivity. One of the challenges to develop this method is the stability of the compounds in human plasma as VNP4090CE has a half-life <90 s. We used different acid concentrations to stabilize VNP40101M and VNP4090CE in the plasma and found that citric acid worked the best at plasma:2.0 M

citric acid = 40:1, *v/v*. Another challenge is that VNP40101M breaks down on the ESI⁻ source to produce the same *m/z* as VNP4090CE; with different retention times we were able to monitor both compounds in one method. Figure 2 shows the LC–MS-MS chromatograms of 10 ng/mL of VNP40101M and VNP4090CE in human plasma, these chromatograms show good separation and clearly indicate the lack of interference from other endogenous.

Method validation for laromustine and its active metabolite in human plasma

Method validation in this work consisted of demonstrating or establishing method selectivity, LOQ, linearity, precision, accuracy, sample carryover, recovery, autosampler viability, freeze–thaw cycle and compound stability.

Selectivity

Aliquots of blank human plasma from six different lots were tested for endogenous interferences. In all cases, the VNP40101M and VNP4090CE and ISTD regions were free from significant interference (<20.0% of the mean utilized LLOQ or <5.0% of ISTD response in the control zero sample). Aliquots of these lots were spiked with 150 ng/mL of VNP40101M and VNP4090CE, including ISTD. The mean concentration of the spiked samples had a percent relative standard deviation (RSD) of ≤15.0% and mean accuracy within the range of 85.0–115.0%. It was therefore concluded that the method demonstrated acceptable selectivity.

To evaluate matrix effect, extracts of blank matrix from the same lots were spiked with 150 ng/mL of VNP40101M and VNP4090CE, including ISTD. In addition, three replicates of a pure solution containing VNP40101M, VNP4090CE and ISTD at the same concentration as the spiked extract were injected. The matrix effect was calculated as the difference between the mean peak area of the spiked blank matrix extract samples and the mean pure solution peak area expressed as a percentage of the mean pure solution peak area. The matrix effect did not significantly impact assay performance. Known isotopic interference of analyte (VNP40101M and VNP4090CE) to respective ISTD (VNP40101M-IS and VNP4090CE-IS) was monitored by injecting samples prepared at the ULOQ without ISTD as system evaluation samples. As determined by the analysis of these samples, it was confirmed that within this assay there is ~1.79% isotopic interference of VNP40101M to VNP40101M-IS and ~3.76% isotopic interference of VNP4090CE to VNP4090CE-IS. Matrix effect data for VNP40101M and VNP4090CE and the ISTD in acidified human plasma are presented in Tables I and II.

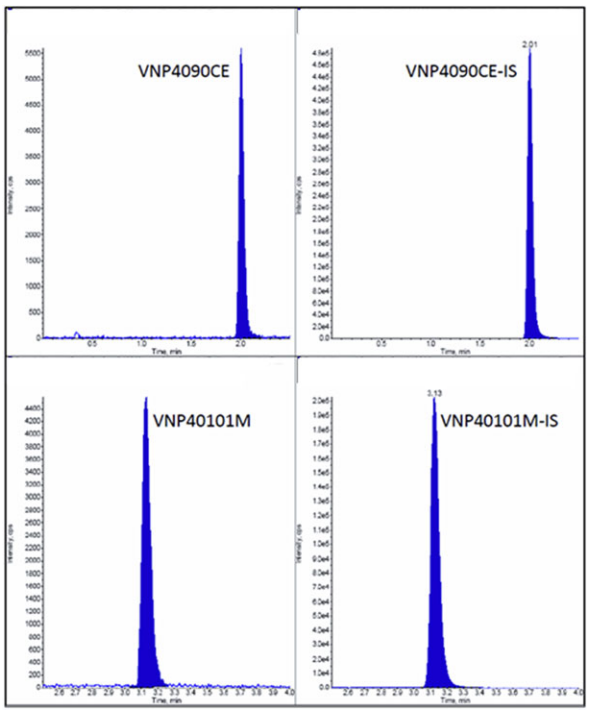

Figure 2. Chromatogram of a 10.0 ng/mL calibration standard: VNP40101M and VNP4090CE in human plasma

Sensitivity

The LLOQ for VNP40101M and VNP4090CE in human plasma was set at 1.00 ng/mL. A method with acceptable sensitivity requires an RSD ≤20.0% and mean accuracy within the range of 80.0–120.0%. In this study, the LLOQ results met these criteria, and it was therefore concluded that the method demonstrated acceptable sensitivity.

Linearity

Results were calculated using peak area ratios. The dynamic ranges for VNP40101M and VNP4090CE in human plasma ranged from 1.00 to 1,000 ng/mL and were generated using a weighted ($1/x^2$) linear least-squares regression. The LLOQ for VNP40101M and VNP4090CE in human plasma was set at 1.00 ng/mL. For VNP40101M, r = 0.9986, slope = 2.11E−03 and y-intercept = 9.67E−04 (Table III). For VNP4090CE, r = 0.9989, slope = 1.04E−03 and y-intercept = 3.00E−04 (Table IV).

Precision and accuracy

The accuracy of the method was determined by comparing the means of the measured concentrations of the calibration standards (inter-assay) and QC samples (intra- and inter-assay) with their theoretical concentrations. The accuracy results of this study demonstrated calculated mean values in the range of 85.0–115.0% (80.0–120.0% at the LLOQ). It was therefore concluded that the method demonstrated was acceptable. The accuracy (%) determinations for calibration standards and LQC, MQC and HQC samples for VNP40101M, n = 18 in acidified human plasma are 102.0, 102.7 and 97.3, respectively. The precision, RSD (%) determinations for calibration standards and LQC, MQC and HQC samples for VNP40101M, n = 18 in acidified human plasma are 4.6, 3.8 and 3.1, respectively.

Sample dilution analysis

The accuracy of data from samples diluted prior to analysis for VNP40101M and VNP4090CE are 99.7 and 98.9, respectively. The precision of data from samples diluted prior to analysis for VNP40101M and VNP4090CE are RSD (%) 1.9 and 1.5, respectively. Data are considered acceptable if the mean concentration has an RSD ≤15.0% and mean accuracy within the range of 85.0–115.0%. Data obtained in this study confirmed the acceptability of dilution prior to analysis.

Table I. Matrix Effect Data for VNP40101M and the ISTD in Acidified Human Plasma

		VNP40101M		ISTD	
Analysis Group	Theoretical concentration (ng/mL)	Post-extraction spike peak area	Pure solution peak area	Post-extraction spike peak area	Pure solution peak area
001	150	318,876.6	311,354.9	967,342.9	936,491.5
		308,068.5	311,607.8	916,504.9	938,876.3
		300,897.6	313,897.5	913,933.0	953,558.1
		308,795.0	910,121.3		
		335,346.0	991,904.2		
		306,624.5	933,726.5		
n		6	3	6	3
Mean	313,101.4	312,286.7	312,286.7	938,922.1	942,975.3
SD	12,352.6	1,400.7	33,461.4	9,242.2	
RSD (%)	3.9	0.4	3.6	1.0	
Matrix effect (%)	0.3			−0.4	

Table II. Matrix Effect Data for VNP4090CE and the ISTD in Acidified Human Plasma

		VNP4090CE		ISTD	
Analysis group	Theoretical concentration (ng/mL)	Post-extraction spike peak area	Pure solution peak area	Post-extraction spike peak area	Pure solution peak area
001	150	319,025.0	306,241.1	1,899,747.4	1,889,732.9
		301,974.8	312,451.5	1,836,420.1	1,860,370.8
		305,444.4	313,278.3	1,803,531.8	1,844,151.3
		306,985.9		1,854,975.8	
		303,252.3		1,858,526.7	
		306,596.3		1,848,559.6	
n		6	3	6	3
Mean		307,213.1	310,657.0	1,850,293.6	1,864,751.7
SD		6,102.6	3,846.5	31,360.9	23,104.4
RSD (%)		2.0	1.2	1.7	1.2
Matrix effect (%)		−1.1		−0.8	

$$\text{Matrix Effect (\%)} = \frac{(\text{Mean post extraction peak area} - \text{Mean pure solution peak area})}{(\text{Mean pure solution peak area})} \times 100$$

Positive value indicates percent enhancement, negative value indicates percent suppression.

Table III. Calibration Curve Parameters for VNP40101M in Acidified Human Plasma

Analysis group	Slope	*y*-Intercept	Correlation coefficient	Analysis date
002	2.07E−03	1.03E−03	0.9993	02 March 2006
003	2.14E−03	5.11E−04	0.9988	03 March 2006
004	2.13E−03	1.36E−03	0.9976	04 March 2006
n	3	3	3	
Mean	2.11E−03	9.67E−04	0.9986	

Table IV. Calibration Curve Parameters for VNP4090CE in Acidified Human Plasma

Analysis group	Slope	*y*-Intercept	Correlation coefficient	Analysis date
002	1.03E−03	2.74E−04	0.9995	02 March 2006
003	1.04E−03	2.15E−04	0.9998	03 March 2006
004	1.06E−03	4.12E−04	0.9973	04 March 2006
n	3	3	3	
Mean	1.04E−03	3.00E−04	0.9989	

Carryover

We checked the possibility of sample carryover from a previous sample by injecting an extract of blank matrix run immediately following the two highest calibration standards; one positioned early in the batch and one toward the end of the batch. The extracts of blank matrix demonstrated a lack of significant carryover in the

chromatographic regions of interest for VNP40101M and VNP4090CE (<20.0% of the mean utilized LLOQ) and ISTD (<5.0% of the ISTD response in the control zero sample), and it was concluded that there was no significant carryover.

Recovery

Recoveries were determined by comparing the mean peak area of extracted LQC, MQC and HQC samples with the mean peak area of recovery samples at concentrations corresponding to the LQC, MQC and HQC samples. Three replicates of each of the recovery samples were prepared by adding VNP40101M and VNP4090CE and ISTD to blank human plasma extracts. The overall recoveries for VNP40101M and the ISTD were 64.1 and 66.1%, respectively. The overall recoveries for VNP4090CE and the ISTD were 71.8 and 75.4%, respectively.

Laromustine PHQC sample

A QC pool was prepared at the HQC level containing parent analyte (VNP40101M; laromustine) only. This QC pool (referred to as the PHQC sample) was analyzed to monitor for the known breakdown of parent analyte (VNP40101M; laromustine) to metabolite (VNP4090CE) in acidified human plasma. Low levels of metabolite (VNP4090CE) were detected in the PHQC samples indicating that conversion of VNP40101M into VNP4090CE does occur. The accuracy of data from PHQC samples, $n = 18$ for VNP40101M is 102.4 and the precision of data from PHQC samples for VNP40101M is RSD (%) 2.7.

Processed-sample (autosampler) viability

Processed-sample viability was tested by extracting a calibration curve and six replicates of LQC, MQC, HQC and PHQC samples, processed and stored refrigerated (5°C) for 52 h. Processed-sample viability was considered acceptable when the mean accuracy at each concentration was within the range of 85.0–115.0%. Processed-sample viability was confirmed for 52 h. During sample analysis, data for extracts stored longer than the established process sample viability may be used if the batch meets acceptance criteria.

Batch size determination

Additional extracted samples (i.e., QC samples or blank matrix) were added to at least one of the validation batches to make the batch size at least as large as any prospective study sample batch. The maximum batch size investigated within this validation was a total of 96 injections (Analysis Group 003). Although this was the maximum analyzed, larger batches may be used during sample analysis if the calibration curve and QC samples in that batch pass acceptance criteria.

The effect of freeze–thaw cycles

For freeze–thaw matrix stability, six replicates of LQC, HQC and PHQC samples were subjected to three freeze–thaw cycles with QC samples stored at −10 to −30°C and with QC samples stored at −60 to −80°C, with samples being frozen for at least 12 h per cycle. Stability was considered acceptable for this study when the mean accuracy at each concentration was within the range of 85.0–115.0%. Stability of VNP40101M and VNP4090CE in human plasma was confirmed for three freeze–thaw cycles for QC samples stored at −10 to −30°C and with QC samples stored at −60 to −80°C.

Short-term matrix stability (4 h)

For room-temperature matrix stability, six replicates of LQC, HQC and PHQC samples were processed after storage at room temperature for 4 h. Stability was considered acceptable for this study when the mean accuracy at each concentration was within the range of 85.0–115.0%. Room-temperature storage stability of VNP40101M and VNP4090CE in human plasma was confirmed for 4 h. For wet-ice matrix stability, six replicates of LQC, HQC and PHQC samples were processed after storage on wet ice for 4 h. Stability was considered acceptable for this study when the mean accuracy at each concentration was within the range of 85.0–115.0%. Wet-ice storage stability of VNP40101M and VNP4090CE in human plasma was confirmed for 4 hours.

Short-term matrix stability (8 and 14 h)

Evaluation of additional room-temperature storage intervals at 8 and 14 h was performed to support unknown study samples potentially stored in excess of the previously established stability of 4 h. For room-temperature matrix stability, six replicates of LQC and HQC samples were processed after storage at room temperature for 8 and 14 h. Stability was considered acceptable for this study when the mean accuracy at each concentration was within the range of 85.0 to 115.0%.

Also, Evaluation of additional wet-ice storage intervals at 8 and 14 hours was performed to support unknown study samples potentially stored in excess of the previously established stability of 4 hours. For wet-ice matrix stability, six replicates of LQC and HQC samples were processed after storage on wet ice for 8 and 14 hours. Stability was considered acceptable for this study when the mean accuracy at each concentration was within the range of 85.0–115.0%. Wet-ice storage stability of VNP40101M and VNP4090CE in human plasma was confirmed for up to 14 h.

Short-term matrix stability

Room-temperature storage stability of VNP40101M in human plasma was confirmed for up to 14 h; results for VNP4090CE failed acceptance criteria, and stability was not confirmed. For the 14-h room-temperature stability evaluation, the VNP40101M low-quality control [LQC (3.00 ng/mL)] stability samples did not meet acceptance criteria but were considered stable based on the following documented discussion:

- LQC room-temperature stability results calculated above theoretical for VNP40101M at 14 h (~116%). If stability was compromised, it would be expected that calculated concentrations would be lower than theoretical.
- Stability LQC samples displayed similar concentrations and area counts as the LQC batch acceptance QC samples. If stability was an issue, it would be expected that calculated concentrations of stability QC samples would be lower than that of batch acceptance QC samples.
- HQC (750 ng/mL) stability samples met defined acceptance criteria at both 8- and 14-h intervals.
- For the 8- and 14-h wet-ice stability evaluation, the VNP40101M LQC (3.00 ng/mL) stability samples did not meet

acceptance criteria but were considered stable based on the following documented discussion:

- LQC stability results calculated above theoretical for VNP40101M at 8 and 14 h (~118 and 119%, respectively). If stability was compromised, it would be expected that calculated concentrations would be lower than theoretical.
- Stability LQC samples displayed similar concentrations and area counts as the LQC batch acceptance QC samples. If stability was an issue, it would be expected that calculated concentrations of stability QC samples (maintained on wet ice for 8 and 14 h) would be lower than that of batch acceptance QC samples.
- HQC (750 ng/mL) stability samples met defined acceptance criteria at both 8- and 14-h intervals.

Frozen matrix stability

Frozen matrix stability of VNP40101M and VNP4090CE in human plasma after storage for 365 days in a freezer set to maintain −10 to −30°C was evaluated. Frozen matrix stability of VNP40101M and VNP4090CE in human plasma after storage for 371 days in a freezer set to maintain −60 to −80°C was evaluated. The frozen QC data were acceptable when the mean accuracy at each concentration was in the range of 85.0–115.0% of theoretical, when calculated against the calibration curve, consisting of viable calibration standards. VNP40101M and VNP4090CE in human plasma were stable for 365 days when stored in a freezer set to maintain −10 to −30°C, and for 371 days when stored in a freezer set to maintain −60 to −80°C before analysis.

Discussion

One of the driving forces for this study was to establish a fast, accurate and sensitive LC–MS-MS procedure for the determination of concentration levels of laromustine and its active metabolite (VNP4090CE) in human plasma. Optimized chromatographic conditions allow greater sensitivity and selectivity to the bioanalytical method. The development and validation of an LC–MS-MS method for unstable drug or prodrug in plasma are always a challenge. VNP4090CE has a half-life in human blood of <90 s; use of citric acid to acidify the human plasma extended the stability of samples to 1 year. Laromustine cleaves on the MS source to give *m/z* 249 which is the same *m/z* for VNP4090CE. Because VNP4090CE and laromustine elute at approximate retention times of 1.93 and 2.94 min, respectively, we successfully quantified both with one method. VNP40101M, VNP4090CE and the ISTD were extracted from human plasma by liquid–liquid extraction into ethyl ether. The ethyl ether layer was evaporated, reconstituted and analyzed using LC with MS/MS detection. The method may be useful for other alkylating agents that are not stable at physiological pH and which do not require an enzyme for simultaneous degradations.

In this study, both intra- and inter-assay results demonstrated an RSD for calibration standards (inter-assay) and QC samples (intra- and inter-assay) to be ≤15.0% (≤20.0% at the LLOQ). It was therefore concluded that the method demonstrated acceptable precision. An extract of blank matrix run immediately following the two highest calibration standards; one positioned early in the batch and one toward the end of the batch. The extracts of blank matrix demonstrated a lack of significant carryover in the chromatographic regions of interest for VNP40101M and VNP4090CE. Based on these results, it was concluded that there was no significant carryover. This method showed good recoveries for VNP40101M and the ISTD were 64.1 and 66.1%, respectively and VNP4090CE and the ISTD were 71.8 and 75.4%, respectively.

As expected, low levels of metabolite (VNP4090CE) were detected in the PHQC samples indicating that conversion of VNP40101M into VNP4090CE does occur. Low levels of conversion should not significantly impact study results; however, it should be noted that analytical results for VNP4090CE samples for which the VNP40101M concentration calculates at 750 ng/mL or greater may be effected by contribution of parent to metabolite by ~0.2%. VNP40101M and VNP4090CE in human plasma were stable for 365 days when stored in a freezer set to maintain −10 to −30°C, and for 371 days when stored in a freezer set to maintain −60 to −80°C before analysis.

Conclusions

A quantitative procedure for the determination of VNP40101M and VNP4090CE in human plasma, over the concentration range of 1.00–1,000 ng/mL, has been successfully validated and proved to be sensitive, specific, linear, precise and accurate. The method utilized a sample volume of 0.100 mL. Frozen matrix stability was established for VNP40101M and VNP4090CE in human plasma for 365 days when stored in a freezer set to maintain −10 to −30°C, and for 371 days when stored in a freezer set to maintain −60 to −80°C before analysis. Short-term matrix stability was confirmed for up to 14 h at room temperature for VNP40101M in human plasma and for up to 14 h on wet ice for VNP40101M and VNP4090CE in human plasma. The method may be useful for other alkylating agents that are not stable at physiological pH and they do not require an enzyme for sonorously degradations. This method was validated, and the mean intra- and inter-day accuracy and precision were in accordance with FDA guidelines.

Supplementary Data

Supplementary material is available at *Journal of Chromatographic Science* online.

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