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Determination of Cloretazine™ (VNP40101M) and its active metabolite (VNP4090CE) in human plasma by liquid chromatography electrospray tandem mass spectrometry (LC–ESI-MS/MS)

Feng Bai [a], Patton Minkin [a], Charles H. Fraga [a], Melinda A. O'Shaughnessy [a], Sri Gururangan [b,c], Clinton F. Stewart [a,d,*]

[a] Department of Pharmaceutical Sciences, St. Jude Children's Research Hospital, Memphis, TN 38105, USA
[b] The Preston Robert Tisch Brain Tumor Center, Duke University Medical Center, Durham, NC 27710, USA
[c] Department of Pediatrics and Surgery, Duke University Medical Center, Durham, NC 27710, USA
[d] Department of Pediatrics, College of Medicine, University of Tennessee, Memphis, TN 38105, USA

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Abstract

A sensitive method for the determination of Cloretazine™ (VNP40101M) and its metabolite (VNP4090CE) with an internal standard (ISTD) in human plasma was developed using high-performance liquid chromatographic separation with tandem mass spectrometric detection. Acidified plasma samples (500 μL) were prepared using solid phase extraction (SPE) columns, and 25 μL of the reconstituted sample was injected onto an Ascentis C_{18} HPLC column (3 μm, 5 cm × 2.1 mm) with an isocratic mobile phase. Analytes were detected with an API-3000 LC–MS/MS System at unit (Q1) and low (Q3) resolution in negative multiple reaction monitoring mode: *m/z* 249.0 (precursor ion) to *m/z* 114.9 (product ion) for both Cloretazine™ (at 3.64 min) and VNP4090CE (at 2.91 min), and *m/z* 253.0 (precursor ion) to *m/z* 116.9 (product ion) for the ISTD. The mean recovery for Cloretazine™ (VNP40101M) and its metabolite (VNP4090CE) was greater than 87% with a lower limit of quantification of 1.0 ng/mL for Cloretazine™ (S/N = 9.7, CV ≤ 12%) and 0.5 ng/mL for VNP4090CE (S/N = 11.3, CV ≤ 9.7%). This method was validated over a linear range of 1.0–1000 ng/mL for Cloretazine™ and 0.5–100 ng/mL for VNP4090CE, and results from a five day validation study demonstrated good within-day and between-day precision and accuracy. This method has been used to measure plasma Cloretazine™ and its metabolite concentrations in a Phase I study in children with recurrent progressive or refractory primary brain tumors.
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Keywords: Plasma; Cloretazine; Liquid chromatography; Electrospray tandem mass spectrometry (LC–ESI-MS/MS)

1. Introduction

Bifunctional DNA alkylating agents, including the chlorethylnitrosoureas, are used to treat a variety of malignancies including lymphomas and brain tumors [1–3]. The primary mechanism of action proposed for these agents is DNA cross-linking through several reactive species with chlorethylating, hydroxyethylating, carbomylating, and vinylating activities [4,5]. However, neither hydroxyethylation nor vinylation of DNA has any known beneficial anti-tumor effects, and hydroxyethylation can be mutagenic to normal cells. In contrast, carbomylation of proteins inhibits repair of DNA alkylation and cross-linking [4–6]. It is conceivable that inhibition of DNA repair may potentiate the cytotoxicity of DNA lesions caused by the cholorethylating species. Therefore, an alkylating agent with chlorethylating and carbomylating activities, but without hydroxyethylating or vinylating properties would be beneficial [7,8]. The sulfonyl hydrazines prodrugs (SHPs) are a new class of DNA alkylating agents that spontaneously generate nucleophilic species with these selective chlorethylating and carbomylating activities.

Cloretazine™ [VP40101M; 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)-2 (methylamino) carbonylhydrazine] is a new sulfonyl hydrazine alkylating agent that demonstrated potential

* Corresponding author at: Department of Pharmaceutical Sciences, St. Jude Children's Research Hospital, 332 N. Lauderdale, Memphis, TN 38105, USA. Tel.: +1 901 495 3665; fax: +1 901 525 6869.
E-mail address: clinton.stewart@stjude.org (C.F. Stewart).

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advantages over existing alkylating agents in preclinical studies [4]. Cloretazine™ first undergoes activation to yield VNP4090CE [1,2-bis(methylsulfonyl)-1-(2-chloroethyl) hydrazine] and a carbamoylating methylisocyanate species. The 90CE rapidly produces a chlorethylating group with an *in vitro* half-life of less than a minute [9]. Cloretazine™ has demonstrated its tolerability in clinical trials in adults with cancer [10–13]. A Phase I pharmacokinetic clinical trial of Cloretazine™ has been conducted in children with recurrent, progressive, or refractory brain tumors. The characterization of Cloretazine™ disposition in this study population required a highly sensitive and specific assay; one that also exhibits sufficient accuracy and precision. However, to date no analytical method (i.e., LC–MS/MS) for the quantitation of both Cloretazine™ and its metabolite (VNP4090CE) in human plasma has been published. Here, we describe a sensitive and specific LC–ESI-MS/MS method with an internal standard for quantification of Cloretazine™ (VNP40101M) and its active metabolite (VNP4090CE) in human plasma.

2. Experimental

2.1. Chemicals

Cloretazine™ (Fig. 1; VNP4010M, 99.1% purity, lot number: 00-03-0022) and its metabolite (Fig. 1; VNP4090CE, 99.0% purity, lot number: VK-183-24) were supplied by Vion Pharmaceuticals, Inc. (New Haven, CT, USA). The internal standard [(1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine (chloroethyl-^{13}C2), 99.0% purity, lot number: PR-16479] was purchased from Cambridge Isotope Laboratories, Inc., Andover, MA, USA. HPLC grade acetonitrile and methanol were obtained from Burdick & Jackson (Muskegon, MI, USA), whereas formic acid (minimum 95%) and ammonium acetate (minimum 97.6%) were purchased from Sigma (St. Louis, MO, USA). Ammonium formate was obtained from Fisher (Fairlawn, NJ, USA). Blank human plasma was obtained from Lifeblood (Memphis, TN, USA). All water was distilled, deionized, and further purified via a Millipore Milli-Q UV plus and Ultra-Pure Water System

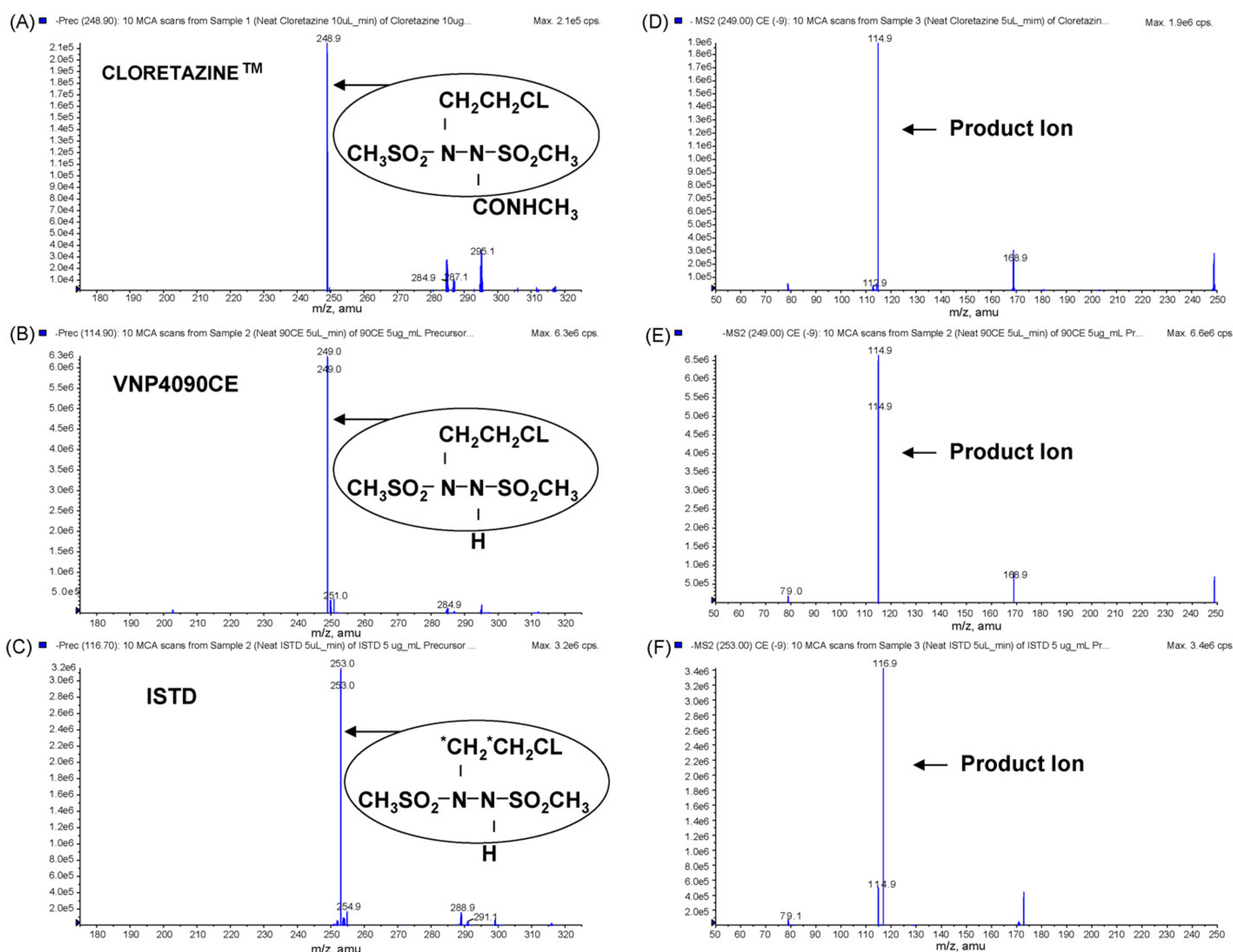

Fig. 1. (A–C) Scan negative precursor ion for the parent compounds of Cloretazine™ and VNP4090CE (both at *m/z* 249.0), and ISTD (at *m/z* 253.0); (D–F) collision induced dissociation spectra for the compounds to the predominant product ion of Cloretazine™ and VNP4090CE (both at *m/z* 114.9), and ISTD (at *m/z* 116.9).

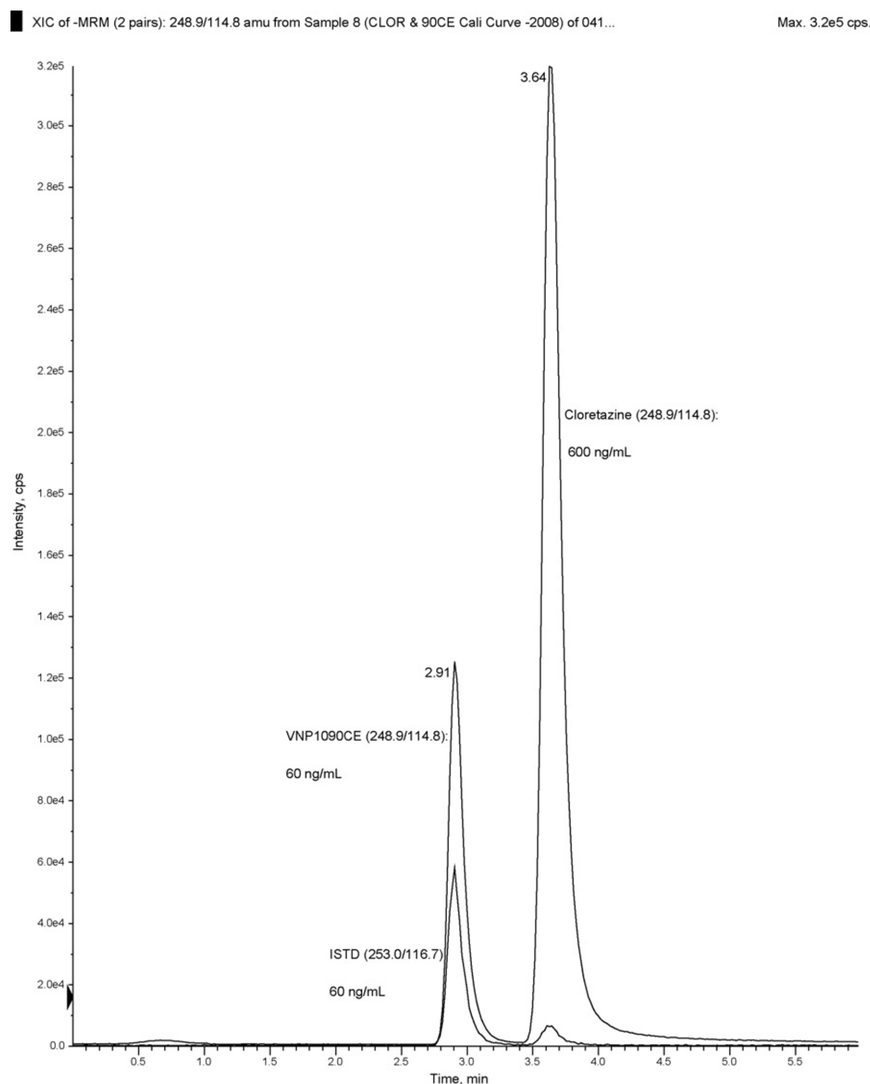

Fig. 2. MRM chromatogram of human plasma spiked with Cloretazine™, VNP4090CE, and ISTD. *Note*: (1) Both Cloretazine™ and VNP4090CE are detected on same mass pair ion 248.9/114.8 and completely separated by HPLC (Cloretazine™ at 3.64 min and VNP4090CE at 2.91 min). (2) VNP4090CE and ISTD are eluted simultaneously at 2.91 min.

(Tokyo, Japan) (resistance 18.2 MΩ). Other chemicals were purchased from standard sources and were of the highest quality available.

2.2. Apparatus and chromatographic conditions

2.2.1. Chromatographic conditions

The HPLC system consisted of a Shimadzu (Kyoto, Japan) system controller (SCL-10AVP), pump (LC-10ADVP), autosampler (SIL-10ADVP), and online degasser (DGU-14A). Reconstituted plasma extract (25 μL) was injected onto a Supelco (Bellefonte, PA, USA) Ascentis C_{18} HPLC column (3 μm, 5 cm × 2.1 mm) preceded by a guard column of the same material (5 μm, 2 cm × 4.0 mm). Flow rate was 250 μL/min with a mobile phase consisting of acetonitrile/3.0 mM ammonium formate (pH 3.5) (30/70, v/v). The column was maintained at 40 °C by HotPocket (Keystone Scientific, Inc., Bellefonte, PA). Under these conditions, the typical retention time was 3.64 min for Cloretazine™ and 2.91 min for both VNP4090CE and ISTD, with a total run time of 4.5 min for each sample (Fig. 2). We observed backpressure values of approximately 56 bar.

2.2.2. Mass spectrometric conditions

Detection was performed with an API 3000 LC–MS/MS System (Toronto, Canada) equipped with a Turbo IonSpray® source (thermally and pneumatically assisted electrospray), which was run at the unit-resolution of Q1 and the low resolution of Q3 in negative mode with multiple reaction monitoring (MRM). Full-scan negative-ion mass spectra (Fig. 1) showed the parent molecular ion for both Cloretazine™ and VNP4090CE at *m*/*z* 249.0 to the predominant ion at *m*/*z* 114.9, and the parent molecular ion for ISTD at *m*/*z* 253.0 to the predominant ion at *m*/*z* 116.9. The optimized conditions of MS/MS with electrospray and HPLC conditions were as follows: ion spray source temperature at 500 °C, nebulizer (NEB) gas at 11, cur-

tain (CUR) gas at 8, turbo gas flow at 7 L/min, ionspray voltage (IS) at −4300 V, and collision-activated dissociation (CAD) at 4.0 units; declustering potential (DP) at −35 V, focusing potential (FP) at −120 V, entrance potential (EP) at −8.0 V, collision energy (CE) at −9.0 V, and collision exit potential (CXP) at −10.0 V. The mass spectrometer was interfaced to a computer workstation running Analyst software (Version 1.4.1 Applied Biosystems, Foster City, CA) for data acquisition and processing.

2.3. *Sample preparation*

2.3.1. *Stock solutions*

Stock solutions were prepared by dissolving Cloretazine™, the metabolite, or the internal standard (ISTD) separately in methanol to yield a concentration of 1.0 mg/mL. Stock solutions were stored at −80 °C, and less than 5% of their nominal values were lost over 5 months. The working solutions for Cloretazine™ (0.1, 1.0, 10.0, and 25.0 μg/mL) and its metabolite (0.1, 1.0, and 5.0 μg/mL) were prepared at the time of assay from the 1.0 mg/mL stock solutions by making dilutions with a solution consisting of methanol/water/formic acid (80/20/0.1, v/v/v). An ISTD working solution (5.0 μg/mL) was prepared from its 1.0 mg/mL stock solution in the same manner.

2.3.2. *Calibration curve and quality controls*

Calibrators were made by adding working solutions of Cloretazine™ and its metabolite to blank human plasma to give final concentrations of Cloretazine™ 1.0, 5.0, 30.0, 100, 200, 400, 600, 800, and 1000 ng/mL and VNP4090CE 0.50, 2.50, 5.0, 10.0, 20.0, 40.0, 60.0, 80.0, and 100 ng/mL with 60.0 ng/mL ISTD. Three plasma controls were prepared in triplicate using the same methodology at concentrations of 2 ng/mL (Control 1), 280 ng/mL (Control 2), and 900 ng/mL (Control 3) Cloretazine™, and 0.8 ng/mL (Control 1), 28 ng/mL (Control 2), and 90 ng/mL (Control 3) VNP4090CE.

2.3.3. *Plasma sample preparation*

A total of 500 μL of spiked plasma or patient plasma was spiked with 6.0 μL ISTD working solution in a 2.0 mL amber microcentrifuge tube. The sample was then acidified with 2.0 μL of formic acid and subsequently treated with 700 μL deionized water. This solution was vortexed and transferred to a vacuum-assisted Phenomenex (San Francisco, CA) Strata-X-CW tube (33 μm, 60 mg/3 mL; PN: 8B-S035-UBJ) preconditioned with 2 × 1 mL methanol washes followed by 2 × 1 mL washes with 25 mM ammonium acetate (pH 6.5). The sample was loaded on a SPE tube and then washed with 4 × 1.0 mL of 25 mM ammonium acetate (pH 6.5). A vacuum was applied for 2 min. The compounds were eluted with 4 × 0.50 mL acidified methanol (containing 2.0% formic acid) and dried under nitrogen gas for 30 min. The sample was reconstituted with 150 μL of 10% acetonitrile in 3.0 mM ammonium formate (pH 3.5), and transferred to a vial from which 25 μL was injected onto the LC–ESI-MS/MS system by an autosampler.

2.3.4. *Patient sample collection and storage*

Patient blood samples (5 mL) were collected in heparinized Vacutainer tubes (Franklin Lakes, NJ) containing 0.125 mL of 2.0 M citric acid solution and then centrifuged at 15,000 × *g* for 15 min to separate the plasma. The plasma was removed and stored at −80 °C until analysis.

2.4. *Ion suppression effects*

Ion suppression was evaluated with a comprehensive approach that uses a post-column infusion of the analyte [14]. Briefly, a neat Cloretazine™ or VNP4090CE sample (100 ng/mL) was infused, post-column, through a Valco zero dead volume tee using a Harvard Apparatus syringe pump 11 (Harvard Apparatus, Holliston, MA, USA) at a constant flow rate of 5.0 μL/min into the LC effluent (200 μL/min) prior to entering the mass spectrometer. Different blank human plasma samples ($n = 3$) and mobile phase solution were then injected onto an Ascentis C_{18} HPLC column. Effluent from the HPLC combined with the infused compound, entered the electrospray interface and was analyzed under the operating conditions for Cloretazine™ or VNP4090CE to measure the "matrix effect," not only from one run, but also from late-eluting compounds that may not be detected until after several sequential analyses had been performed.

2.5. *Assay validation*

The method developed for the quantitation of Cloretazine™ and its metabolite in human plasma was validated over five days by analysis of plasma quality control samples, and the within-day and between-day precision and accuracy for the method were determined. Two calibration curves were analyzed. The linear regression of the ratio of both Cloretazine™/ISTD and VNP4090CE/ISTD peak areas were weighted by $1/x$. The coefficients of determination (R^2) were used to evaluate the linearity of both calibration curves.

The limit of quantitation (LOQ) was defined as the minimum value at which the ratio of signal/noise was ≥10. This was determined by triplicate analysis of an extensive calibration curve in the low concentration range (0.5–2.0 ng/mL).

The stability of Cloretazine™ and VNP4090CE at 4 °C and room temperature (25 °C) in both plasma and reconstituted extracted plasma for up to 24 h, and at −80 °C for two months were evaluated at two concentrations (Controls 1 and 3) for both analytes.

The freeze-thaw stability of Cloretazine™ and VNP4090CE at −80 °C was evaluated over three freeze-thaw cycles at two concentrations (Controls 1 and 3) for both analytes.

2.6. *Application of method to patient samples*

Serial blood samples (5 ml) were collected from a pediatric patient after a 30 min intravenous infusion of 30 mg/m^2 of Cloretazine™. Each blood sample was collected in acidified heparinized vacutainer tubes (Franklin Lakes, NJ). Samples

were reconstituted and then analyzed using the described method.

3. Results and discussion

3.1. Chromatography

Since the same mass ion pairs (*m/z*: 248.9/114.8) were selected on the MS/MS spectrometer for both Cloretazine™ and its metabolite (VNP4090CE), the two compounds had to be completely separated by HPLC for their accurate quantitation. Based on their chemical characteristics and a baseline separation of the two compounds within 5 min, we tested two HPLC short columns in early method development: Ascentis C_{18} HPLC column (3 μm, 5 cm × 2.1 mm) and Synergy Fusion-RP column (2 μm, 50 × 2.00 mm). Following optimization of HPLC conditions to increase mass signal strength for both compounds in terms of counts per second (cps), the Ascentis C_{18} column improved both separation and retention of compounds when compared to the Synergy Fusion-RP column, with a moderate backpressure (56 bar). Optimal improvement in signal response and peak separation was achieved when the mobile phase composition was adjusted to decrease both the percentage of acetonitrile from 60% to 30% and the pH of ammonium formate buffer (3.0 mM) from 5.0 to 3.5. Column temperatures of 25 °C, 30 °C, 40 °C, and 50 °C were evaluated. A column temperature of 40 °C resulted in an optimal signal response and a baseline separation with an acceptable sample running time (5.0 min). According to ions intensity (cps), peak shape, and separation of both Cloretazine™ and VNP4090CE with ISTD, the Ascentis C_{18} HPLC column best performed at 40 °C with a mobile phase consisting of acetonitrile/3.0 mM ammonium formate buffer (30/70, v/v) at pH 3.5. The retention times of VNP4090CE, ISTD, and Cloretazine™ were 2.91, 2.91, and 3.64 min, respectively (Fig. 2; plasma Cloretazine™ and VNP4090CE with ISTD).

3.2. Plasma sample preparation

Initially, proteins were precipitated from spiked plasma samples with cold methanol (plasma/methanol = 200/800, v/v). The collected supernatant was dried by nitrogen gas and subsequently reconstituted in the mobile phase. Both Cloretazine™ and VNP4090CE had a noisy baseline with low recovery (the cps were 20–30% less than the neat samples at the same concentration). Since Cloretazine™ is a basic compound with pK_a of approximately 8.0, it can be retained on SPE with a cation exchange stationary phase [15–17]. During development of a SPE extraction method for Cloretazine™, VNP4090CE, and ISTD in plasma, we evaluated two different SPE extracting conditions on Phenomenex Strata-X-CW (weaker cation exchange). The Strata-X-CW tube was first equilibrated with methanol, and then conditioned with either distilled water or a 25 mM ammonium acetate buffer (pH 6.5) before loading the sample. The spiked plasma samples were pretreated with formic acid (plasma/HCOOH = 500/2, v/v) to positively charge all analytes

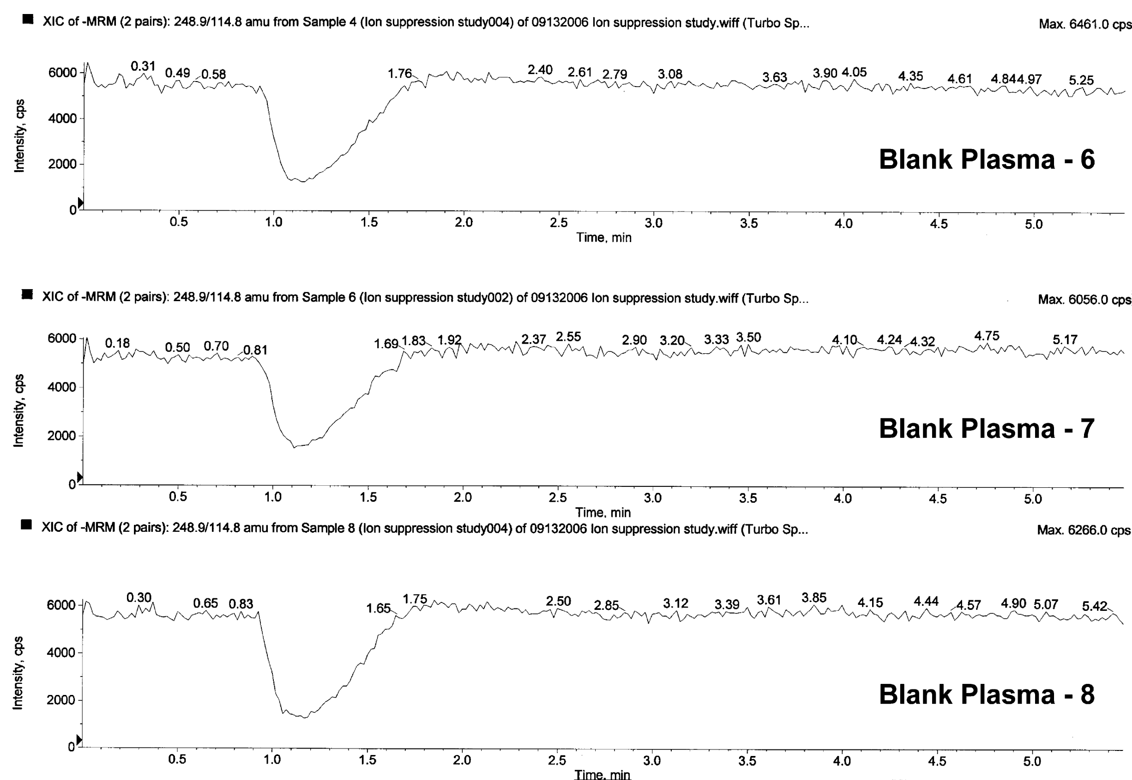

Fig. 3. Infusion chromatogram for both Cloretazine™ and VNP4090CE – a neat mixture of Cloretazine™ and VNP4090CE (100 ng/mL) was constantly infused, post-column, at a flow rate of 5.0 μL/min into the LC effluent: (A) blank human plasma 6, (B) blank human plasma 7, and (C) blank human plasma 8.

before loading the sample. SPE tubes were then washed with either distilled water or 25 mM ammonium acid buffer (pH 6.5) and eluted with methanol/formic acid (100/2, v/v). The collected eluant was dried under nitrogen and reconstituted with 10% acetonitrile in 3.0 mM ammonium formate at pH 3.5. Average recovery of Cloretazine™, VNP4090CE, and ISTD was greater in the Strata-X-CW when buffer was used during the washing steps (90%, 84%, and 76%, respectively) than when distilled water was used (80%, 70%, and 65%, respectively).

3.3. Ion suppression

Ion suppression is an important factor affecting the quantitative performance of a mass detector, especially when using an electrospray interface [18]. Ion suppression effect on both Cloretazine™ and VNP4090CE from SPE extracted human blank plasma ($n = 3$) were evaluated. Depicted in Fig. 3 are the results from these studies that illustrate the response obtained from the post-column applied infusion. Since Cloretazine™ and VNP4090CE with ISTD eluted between 2.0 and 4.0 min in this method, a mixture of Cloretazine™ and VNP4090CE at 100 ng/mL was infused in this ion suppression study. The results demonstrated the absence of either relevant suppression or enhancement of Cloretazine™ and VNP4090CE ion intensity at retention times of 3.64 and 2.91 min, respectively (Fig. 2), and in the relative time range of 1.75–6.00 min in this LC–MS/MS method.

3.4. Assay validation

To assess within-day and between-day precision and accuracy, we evaluated validation parameters for Cloretazine™ and VNP4090CE (Table 1). Ten injections of low, medium, and high concentration Cloretazine™ and VNP4090CE control samples were made on days one and two to assess within-day variability and again on days three and four to evaluate between-day variability. The LLOQs in plasma for this method were 0.5 ng/mL (S/N = 11.3, $n = 3$, CV ≤ 9.7%) for VNP4090CE and 1.0 ng/mL for Cloretazine™ (S/N = 9.7, $n = 3$, CV ≤ 12%), respectively. The average recovery of plasma Cloretazine™ (1.0, 500, and 1000 ng/mL in triplicate) and VNP4090CE (0.5, 50, and 100 ng/mL in triplicate) in comparison to their neat samples at the same concentrations were 90% for Cloretazine™ ($n = 3$, SD = 3.06, CV = 4.09%) and 84% for VNP4090CE ($n = 3$, SD = 1.25, CV = 3.22%). The plasma calibration curves were linear from 1.0 to 1000 ng/mL for Cloretazine™ and 0.5 to 100 ng/mL for VNP4090CE, with correlation coefficients (R^2) greater than 0.998.

Table 1
Validation parameters of Cloretazine™/VNP4090CE in human plasma

Plasma CLOR/90CE (ng/mL)	Within-day ($n = 10$)		Between-day ($n = 5$)	
	%RSD	%Error	%RSD	%Error
2.0/0.8	3.1/4.3	−5.2/3.5	4.8/6.2	−0.2/−5.2
280/28	1.9/1.4	5.2/5.0	5.3/4.5	1.7/0.1
900/90	7.0/3.7	−2.5/−0.9	7.3/6.4	1.7/−4.1

Ten injections of low, medium, and high quality control of plasma Cloretazine™/VNP4090CE samples (CLOR/90CE) were run within one day to assess within-day variability and 15 injections were run within five days to evaluate between-day variability. Variability reported as relative standard deviation (%RSD) and percentage error (%Error).

3.5. Stability

To assess the short-term stability of reconstituted Cloretazine™ and VNP4090CE extract, low and high concentrations of Cloretazine™ (2 and 900 ng/mL) and VNP4090CE (0.8 and 90 ng/mL) were evaluated at 4 °C and 25 °C for 24 h after solid phase extraction and reconstitution in acetonitrile and 3 mM NH4COOH at a pH of 3.5 (10/90, v/v). As depicted in Table 2a, we observed less than a 6.8% decrease

Table 2a
Stability of Cloretazine™ and VNP4090CE in SPE extracted human plasma

Plasma (SPE extraction)	Cloretazine™		VNP4090CE	
	Control 1	Control 3	Control 1	Control 3
<1 h	14033 (1.28)[a]	6643333 (1.52)	48167 (1.28)	5820000 (1.52)
4 °C				
6 h	13200 (1.23)	6193333 (1.43)	45800 (1.23)	5430000 (1.43)
%change	−5.9	−6.8	−4.9	−6.7
24 h	11120 (0.99)	5526667 (1.20)	36767 (0.99)	4673333 (1.20)
%change	−20.8	−16.8	−23.7	−19.7
25 °C				
6 h	12100 (1.05)	5476667 (1.19)	40000 (1.05)	4493333 (1.19)
%change	−13.8	−17.6	−17.0	−22.8
24 h	10866 (0.82)	5026666 (0.79)	27566 (0.82)	3146666 (0.79)
%change	−22.6	−24.3	−42.8	−45.9

The reconstituted SPE extracted plasma Cloretazine™ and VNP4090CE samples at two concentrations with internal standard were separately stored (in triplicate) at 4 °C and 25 °C, then analyzed up to 24 h. Data are expressed as mean percent of initial peak area of three samples tested. Data are expressed as the mean of the peak areas for each sample, and percent change between the peak areas for samples at each time point and the initial peak areas (<1 h) are expressed for Cloretazine™ and VNP4090CE.

[a] Value in parentheses represents the peak area for the internal standard expressed as $\times 10^6$.

Table 2b
Stability of Cloretazine™ and VNP4090CE in human plasma

Plasma ($n=3$)	Cloretazine™		VNP4090CE	
	Control 1	Control 3	Control 1	Control 3
<1 h	2.05	1083	0.73	85.7
4 °C				
6 h	2.01	987	0.73	83.4
%change	−2.1	−8.9	0.1	−2.7
24 h	1.95	983.7	0.70	78.0
%change	−4.7	−9.2	−4.5	−9.0
25 °C				
6 h	1.86	982	0.66	77.0
%change	−9.1	−9.3	−10.0	−10.1
24 h	1.65	895.3	0.3	37.6
%change	−19.3	−17.4	−65.0	−56.2
−80 °C				
Day-10	2.03	1050	0.74	87.7
%change	−1.0	−3.1	0.9	2.3
Day-30	1.98	999.0	0.8	82.6
%change	−3.4	−7.8	2.3	−3.6
Day-62	2.00	1070.0	0.7	83.3
%change	−2.4	−1.2	−3.2	−2.8

Spiked plasma samples with high and low of Cloretazine™ and VNP4090CE concentrations were aliquotted in triplicate and separately stored at 4 °C, 25 °C, and −80 °C. Before analysis, the samples were spiked with ISTD first and then extracted by SPE. Stability was assessed by each measured concentration. Data presented is mean percent of initial concentration (<1 h) of three samples tested.

in both Cloretazine™ and VNP4090CE peak areas at 4 °C for 6 h. However, the percentage decrease of the peak areas for both compounds at 4 °C for 24 h and at 25 °C ranged from 13.8% to 45.9%. Thus, both Cloretazine™ and VNP4090CE are considered stable in reconstituted SPE extracted plasma samples at 4 °C for 6 h; however, they are only stable at 25 °C for 1 h.

To test the short-term stability of both Cloretazine™ and VNP4090CE in plasma at 4 °C and 25 °C as well as the long-term stability at −80 °C, we evaluated two concentrations of Cloretazine™ (2 and 900 ng/mL) and VNP4090CE (0.8 and 90 ng/mL) stored at 4 °C and 25 °C for 24 h and −80 °C for two months. Before extraction and subsequent analysis, ISTD was added to the samples. As depicted in Table 2b, the plasma concentrations of Cloretazine™ and VNP4090CE decreased less than 10% when stored at 4 °C for 24 h, 25 °C for 6 h, and −80 °C for two months.

To evaluate the freeze-thaw stability of Cloretazine™ and VNP4090CE in plasma at −80 °C, we evaluated two concentrations of Cloretazine™ (2 and 900 ng/mL) and VNP4090CE (0.8 and 90 ng/mL) that were stored at −80 °C overnight. The next day samples were thawed at room temperature for 15–20 min, an aliquot from each of the samples was processed with SPE, and analyzed on the LC–MS/MS in duplicate. The remaining unprocessed samples were placed back in the freezer for subsequent freeze-thaw tests. This was done for three cycles of freeze-thaw. The results of this experiment showed that after three cycles of freeze-thaw the cloretazine controls had <1% change from baseline, whereas the 90CE controls had 11% loss compared with baseline.

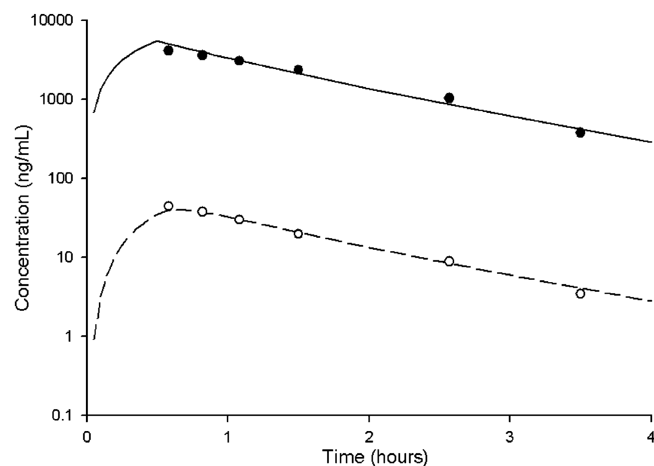

Fig. 4. Cloretazine™ and VNP4090CE plasma concentration–time profile in a pediatric patient after one IV dose of Cloretazine™ (30 mg/m^2). Plasma Cloretazine™ concentration–time points (●) are plotted and the solid line represents the best-fit curve resulting from the pharmacokinetic analysis. Plasma VNP4090CE concentration-time points (○) are plotted and the dashed line represents the best-fit curve resulting from the pharmacokinetic analysis.

3.6. Application of assay in patient blood sample

Plasma samples from a patient enrolled on a Phase I pharmacokinetic trial of Cloretazine™ were analyzed to demonstrate the applicability of the method. Serial plasma samples were collected, processed, and analyzed according to the methods described in this report. A representative plasma concentration-time profile for both Cloretazine™ and VNP4090CE after intravenous Cloretazine™ administration is depicted in Fig. 4. This method was developed to quantitate Cloretazine™ and VNP4090CE in human plasma samples and possesses sufficient precision (CV ≤ 7.3%) and accuracy (−5 ≤ %Error ≤ 5.0), for application in clinical studies of Cloretazine™ pharmacokinetics in both pediatric and adult populations.

4. Conclusion

We report here a LC–ESI-MS/MS method for the rapid and precise quantitation of Cloretazine™ and its metabolite, VNP4090CE in human plasma samples. This method is sensitive and specific, which further enhances its utility as an analytical method for use in clinical pharmacokinetic studies of Cloretazine™ and VNP4090CE. Moreover, given the sensitivity of this method (S/N ≥ 10 at 0.5 ng/mL for VNP4090CE and 1.0 ng/mL for Cloretazine™, respectively), it may also be useful for *in vitro* studies of Cloretazine™ (e.g., tissue culture studies) where low concentrations or small sample volumes may be expected. In addition, by using a narrow-bore short column instead of 4.6 id column with lower flow rate, this assay reduces solvent costs and minimizes environmental impact of the toxic solvent. Finally, we have successfully applied this LC–ESI-MS/MS method by measuring Cloretazine™ and VNP4090CE in human plasma from a clinical pharmacokinetic study in a child treated with intravenous Cloretazine™.

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References

[1] N.I. Nissen, T.F. Pajak, O. Glidewell, J. Pedersen-Bjergaard, L. Stutzman, G. Falkson, J. Cuttner, J. Blom, L. Leone, A. Sawitsky, M. Coleman, F. Haurani, C.L. Spurr, J.B. Harley, B. Seligman, C. Cornell Jr., P. Henry, H.J. Senn, K. Brunner, G. Martz, P. Maurice, A. Bank, L. Shapiro, G.W. James, J.F. Holland, Cancer 43 (1979) 31.
[2] K.A. Jaeckle, H.J. Eyre, J.J. Townsend, S. Schulman, H.M. Knudson, M. Belanich, D.B. Yarosh, S.I. Bearman, D.J. Giroux, S.C. Schold, J. Clin. Oncol. 16 (1998) 3310.
[3] Z.P. Chen, D. Yarosh, Y. Garcia, D. Tampieri, G. Mohr, A. Malapetsa, A. Langleben, L.C. Panasci, Can. J. Neurol. Sci. 26 (1999) 104.
[4] R.A. Finch, K. Shyam, P.G. Penketh, A.C. Sartorelli, Cancer Res. 61 (2001) 3033.
[5] K. Shyam, L.A. Cosby, A.C. Sartorelli, J. Med. Chem. 28 (1985) 525.
[6] P.G. Penketh, K. Shyam, R.P. Baumann, J.S. Remack, T.P. Brent, A.C. Sartorelli, Cancer Chemother. Pharmacol. 53 (2004) 279.
[7] R.P. Baumann, H.A. Seow, K. Shyam, P.G. Penketh, A.C. Sartorelli, Oncol. Res. 15 (2005) 313.
[8] K. Ishiguro, H.A. Seow, P.G. Penketh, K. Shyam, A.C. Sartorelli, Mol. Cancer Ther. 5 (2006) 969.
[9] P.G. Penketh, K. Shyam, A.C. Sartorelli, Biochem. Pharmacol. 59 (2000) 283.
[10] F. Giles, D. Rizzieri, J. Karp, N. Vey, F. Ravandi, S. Faderl, K.K. Dad, G. Verhoef, P. Wijermans, A. Advani, G. Roboz, H. Kantarjian, B.S. Fazl Ali, A. Ferrant, S.M. Daenen, V. Karsten, A. Cahill, M. Albitar, G. Mufti, S. O'Brien, J. Clin. Oncol. (2006).
[11] F. Giles, S. Verstovsek, S. Faderl, N. Vey, J. Karp, G. Roboz, K.D. Khan, M. Cooper, S.F. Bilgrami, A. Ferrant, S. Daenen, V. Karsten, A. Cahill, M. Albitar, H. Kantarjian, S. O'Brien, E. Feldman, Leuk. Res. 30 (2006) 1591.
[12] F. Giles, S. Verstovsek, D. Thomas, S. Gerson, J. Cortes, S. Faderl, A. Ferrajoli, F. Ravandi, S. Kornblau, G. Garcia-Manero, E. Jabbour, S. O'Brien, V. Karsten, A. Cahill, K. Yee, M. Albitar, M. Sznol, H. Kantarjian, Clin. Cancer Res. 11 (2005) 7817.
[13] F. Giles, D. Thomas, G. Garcia-Manero, S. Faderl, J. Cortes, S. Verstovsek, A. Ferrajoli, S. Jeha, M. Beran, C. Koller, M. Andreeff, A. Cahill, C. Clairmont, M. Sznol, H. Kantarjian, Clin. Cancer Res. 10 (2004) 2908.
[14] T.M. Annesley, Clin. Chem. 49 (2003) 1041.
[15] T.R. Krishnan, I. Ibraham, J. Pharm. Biomed. Anal. 12 (1994) 287.
[16] S. Huq, A. Dixon, K. Kelly, K.M. Kallury, J. Chromatogr. A 1073 (2005) 355.
[17] P.H. Roberts, P. Bersuder, J. Chromatogr. A 1134 (2006) 143.
[18] R. King, R. Bonfiglio, C. Fernandez-Metzler, C. Miller-Stein, T. Olah, J. Am. Soc. Mass Spectrom. 11 (2000) 942.