Published in final edited form as:

Biomed Chromatogr. 2015 November ; 29(11): 1708–1714. doi:10.1002/bmc.3483.

A sensitive liquid chromatography–mass spectrometry bioanalytical assay for a novel anticancer candidate – ZMC1

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Abstract

ZMC1 {azetidinecarbothioic acid, [1-(2-pyridinyl) ethylidene] hydrazide} is a lead compound being developed as one of the first mutant p53 targeted anti-cancer drugs. Establishing a precise quantitative method is an integral component of this development. The aim of this study was to develop a sensitive LC/MS/MS assay suitable for assessing purity, stability and preclinical pharmacokinetic studies of ZMC1. Acetonitrile protein precipitation extraction was chosen for plasma sample preparation with satisfactory recovery (84.2–92.8%) for ZMC1. Chromatographic separation was achieved on an Xterra C₁₈ column (50 × 4.6 mm, 3.5 μm) using a gradient elution with mobile phase of 0.1% formic acid in water and acetonitrile. ZMC1 and internal standard 2-amino-6-bromobenzothiazole were identified using selected-ion monitoring mode at *m/z* 235.2/178.2 and *m/z* 231.0/150.0 at retention times of 5.2 and 6.3 min, respectively. The method was validated with a linearity range of 3.9–500.0 ng/mL in human plasma and showed acceptable reproducibility with intra- and interday precisions <5.9 and 10.5%, and accuracy within ±5.4% of nominal values. This analytical method together with basic stability data in plasma and plasma binding experiments provides a reliable protocol for the study of ZMC1 pharmacokinetics. This will greatly facilitate the pre-clinical development of this novel anti-cancer drug.

Keywords

thiosemicarbazone; LC-MS/MS; plasma stability; protein binding

Introduction

In the era of targeted molecular therapy for cancer three of the most commonly mutated genes, RAS, MYC and TP53, remain without effective agents. TP53 is the most commonly

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mutated gene in human cancer with the majority of mutations being mis-sense mutations in which a protein is found at high levels in cancer cells that is defective for wildtype transcriptional function, making TP53 the most significant tumor suppressor in cancer. Restoring wildtype structure and function to mutant p53 is considered one of the ‘holy grails’ of developmental therapeutics. Recently two small-molecule compounds (NSC319725, NSC319726) were identified from the NCI60 anti-cancer drug screen as having particular sensitivity in human cancer cell line expression mutant p53 (Yu *et al.*, 2012a). NSC319726 was found to reactivate the p53-R175H mutant by restoring wildtype structure and function to mutant p53. The p53-R175H mutant is the most common mis-sense mutant found in human cancer. Both NSC319725 and NSC319726 belong to the thiosemicarbazone family of metal ion chelators, and have received considerable research interest for their wide range of pharmacological activity, including antibacterial, anti-viral and anti-neoplastic (Yu *et al.*, 2009). This family of compounds exhibiting anti-proliferative activity in current literature includes 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (Triapine; Wadler *et al.*, 2004), 2-benzylpyridine 4, 4-dimethyl-3-thiosemicarbazone (Yu *et al.*, 2011, 2012b) and di-2-pyridylketone-4, 4-dimethyl-3-thiosemicarbazone (Dp44mT; Whitnall *et al.*, 2006; Potuckova *et al.*, 2014; Quach *et al.*, 2012). The anti-proliferative effects of these chelators has been attributed to their iron-chelating properties in which they inhibit the iron-dependent enzyme ribonucleotide reductase as well as generating hydroxyl free radicals through Fenton chemistry (Merlot *et al.*, 2013). NSC319726 has recently been named ZMC1 as the mechanism of action for this compound has been reported to involve a zinc metallochaperone function (Yu *et al.*, 2014). The p53-R175H mutant has impaired zinc binding as its principle defect. ZMC1 restores wildtype structure and function to this mutant by delivering zinc to protein to allow it to refold properly. In addition, the compound induces reactive oxygen species levels that serve to transactivate the mutant protein through post-translational modifications.

Progress in the development of these drugs as novel anti-cancer drugs requires modern analytical methodologies appropriate for the qualitative and quantitative evaluation. The analytical method is important to achieve reliable chemical and stability data on the candidates and further provide solid resources for the pharmaceutical formulations. Despite the promising anti-proliferative efficacy of the thiosemicarbazone compounds, few analytical methods have been reported including HPLC with UV detection for purity and stability for pharmacokinetic studies for Triapine and Dp44mT (Stariat *et al.*, 2009; Schelman *et al.*, 2009; Debebe *et al.*, 2012). LC/MS/MS was reported for metabolites and pharmacokinetics studies for the thiosemicarbazone Bp4eT in rats. (Stariat *et al.*, 2014). So far, no relative analytical method is available in the literature for ZMC1 quantitation. Owing to the poor absorption in the UV wavelength region and no fluorescence functional group of ZMC1, an LC/MS/MS assay was first developed and validated for the chemical properties, stability and preclinical pharmacokinetic evaluation of ZMC1. The stability of ZMC1 in different solvents and biological matrices was also investigated in this study.

Experimental

Chemicals

ZMC1 was provided by the Dr. Carpizo Laboratory (>98% by HPLC). Internal standard 2-amino-6-bromobenzothiazole (ABBT) was purchased from Matrix Scientific Inc. (Columbia, SC, USA). HPLC-grade solvents methanol and acetonitrile were purchased from J. T. Baker (Pittsburg, PA, USA). Formic acid was purchased from EMD Millipore (Bedford, MA, USA). Human serum albumin (HSA), human α_1 -acid glycoprotein (AAG) and EDTA (0.5 M in water, pH 8.0) were purchased from Sigma Adrich Co (St Louis, MO, USA). Deionized water was prepared using a Milli-Q purification system (Bedford, MA, USA). Analytical-grade reagents were used throughout the experiment. The control plasma used for the preparation of calibration standards was obtained from the New Brunswick affiliated hospital blood bank (New Brunswick, NJ, USA).

Preparation of standard solutions, calibration samples and quality control samples

A stock solution of ZMC1 was prepared in DMSO (100 mM) and stored at -70°C . The ABBT stock (1.0 mg/mL) was prepared in methanol and diluted to 10 $\mu\text{g/mL}$ as working IS. A set of calibration standards of ZMC1 at concentrations of 1.9, 3.9 7.8 15.6, 31.3, 62.5, 125, 250 and 500 ng/mL were prepared in human plasma and in a solvent of acetonitrile–water (80:20, v/v) by serial dilution of stock solutions. The plasma standard, solvent standard and internal standard were stored at -20°C .

Plasma sample extraction procedure

Plasma ZMC1 concentrations were determined using acetonitrile deproteinization method. Sample aliquots (50 μL standards or samples) were spiked with 20 μL of an internal standard solution and 4 μL EDTA (0.5 M) and the proteins were precipitated with 150 μL acetonitrile. After vortex-mixing at 3000 rpm for 30 s, the sample was centrifuged at 13,000 rpm for 8 min and 25 μL of the supernatant was analyzed by LC-MS/MS system.

Instrumentation and operating conditions

Chromatographic separation of ZMC1 and internal standard was performed using an Xterra C₁₈ column, 50×4.6 mm, 3.5 μm (Waters, MA, USA) at a column temperature of 25°C . The flow rate used was 250 $\mu\text{L/min}$ with mobile phase of 0.1% formic acid (mobile phase A) and acetonitrile with 0.1% formic acid (mobile phase B). Gradient linear elution was as follows: 0–5.5 min, 95–50% A; 5.5–6.0 min, 50–10% A; 6.0–8.0 min, 10–95% A; 8.0–10.0 min, 95% A. The retention times of ZMC1 and IS were 5.2 and 6.3 min respectively.

Quantitation of ZMC1 was performed using Thermo Electron TSQ Quantum mass spectrometer equipped with an electrospray ionization (ESI) source operated in positive ion mode, a Surveyor MS pump and a Surveyor autosampler (Thermo Electron, San Jose, CA, USA). MS/MS conditions were as follows: ion spray voltage, 5000 V; capillary temperature, 350°C ; sheath gas pressure, 5 arbitrary units; Aux gas pressure, 30 arbitrary units; and collision pressure, 1.5 arbitrary units. ZMC1 and IS were detected in single reaction monitoring mode, m/z 235.2/178.0 (ZMC1) and m/z 231.0/150.0 (ABBT). Chromatographic

data acquisition, peak integration and quantitation were performed using Xcalibur software (Thermo Electron, San Jose, CA, USA).

Method validation

The validation was performed following the guidelines for bioanalytical method validation published by the US Food and Drug Administration (<http://www.fda.gov/downloads/Drugs/Guidances/ucm070107.pdf>). The LC/MS/MS method was fully validated for specificity, sensitivity, matrix effect, recovery, linearity, precision, accuracy and stability as described in detail below.

Specificity and calibration curve—The specificity of the method was evaluated by analyzing four different batches of human control plasma and comparing them with the corresponding plasma samples spiked with ZMC1 and IS for the exclusion of any endogenous co-eluting interferences. There was no background measured for the MS/MS transition chosen. Calibration curves of eight different concentration levels were prepared in triplicates and peak area ratio vs concentration was fitted using simple least square linear regression with weight factor of 1/concentration.

Precision and accuracy—Precision and accuracy were evaluated at three concentration levels (3.9, 31.3 and 500 ng/mL) of quality control plasma samples. The intraday precision and accuracy were measured by analysis of three aliquots of each quality control sample on the day. The quality controls samples were measured on four different days for interday precision and accuracy evaluation. The measured concentrations were calculated using the calibration curve. The precision of the assay was calculated at each concentration and expressed as the RSD (Relative standard deviation). The accuracy was compared between the measured value and nominal concentration of analyte expressed as bias (%). Acceptable criteria included precision with RSD <15% and bias within ±15% of nominal concentration, except for the LLOQ, where the limits was RSD <20% and ±20% for accuracy.

Recovery and matrix effect—For matrix effect and recovery studies, three series of QC samples were prepared in duplicate in solvent (acetonitrile–water, 80:20, v/v), plasma acetonitrile deprotein matrix and plasma spiked with internal standard. Absolute recovery and matrix effects were calculated as follows (Matuszewski et al., 2003):

$$\text{RE (\%)} = (\text{Response}_{\text{extracted sample}} / \text{Response}_{\text{non-extracted solvent standard}}) \times 100;$$
$$\text{Matrix effects (\%)} = (\text{Response}_{\text{post-extracted spiked sample}} / \text{Response}_{\text{non-extracted solvent standard}}) \times 100$$

Stability—The stability of ZMC1 in plasma and different solvents (acetonitrile–water 80:20; PBS, (phosphated buffered saline) 0.1 m HCL and 0.1 m NaOH) was analyzed at 4 and 37 °C and room temp for 24 h. The experiments to determine short- and long-term stability of ZMC1 in plasma were performed at three QC concentrations as per the bioanalytical guidelines, keeping the samples at 4 °C for 2 h and –80 °C for 1 month followed by quantitation of ZMC1 using the analytical method. Three freeze–thaw cycle stability was also performed at room temperature on the bench top, not protected from light, using QC samples. The post-preparative stability test was assessed by determining the

processed samples kept in the autosampler for 24 h at room temperature. The analyte was considered to be stable in plasma when 85–115% of the initial concentration was found.

ZMC1 plasma binding experiments

ZMC1 binding to plasma proteins/matrix molecules was determined by ultrafiltration and ultracentrifugation methods (Barré et al., 1985). For ultra-filtration method, pooled human plasma was spiked with ZMC1 in DMSO to a final concentration of 500 ng/mL and incubated for 1 h at 37 °C. The spiked plasma sample was centrifuged in an ultrafiltration device with a 30 kDa cutoff (Microcon[®], EMD Millipore Co., Billerica, MA, USA) for 20 min at 13,000 *g*. For ultracentrifugation, the same spiked plasma was centrifuged at 50,000 rpm with fixed-angle rotor 50Ti for 10 h at 20 °C. (Beckman). The total concentration in plasma (C_{total}), ultrafiltrate concentration and ultra centrifugation supernatant (C_{free}) were measured by LC/MS/MS as described above. The plasma protein binding was calculated using the following formula:

$$\% \text{ bound} = 100 \times (C_{\text{total}} - C_{\text{free}}) / C_{\text{total}}$$

For ultrafiltration method, the recovery of ZMC1 from the water was calculated to correct the nonspecific adsorption on the ultrafiltration membrane. To investigate the contribution of plasma protein binding of ZMC1, 4% human serum albumin and 0.1% human α_1 -acid glycoprotein spiked with 500ng/mL ZMC1 were prepared in PBS buffer following the ultrafiltration method.

Results and discussion

LC-MS/MS method development and optimization

The chemical structure of ZMC1 and internal standard ABBT is presented in Fig. 1. The selection of internal standard is critical because it influences repeatability, reproducibility and accuracy using an electrospray ion source. The unavailability of a stable isotope-labeled ZMC1 led to the choice of chemical ABBT as the internal standard and to optimize the gradient elution to achieve the complete separation from ZMC1 (Fig. 2). ZMC1 is a weak acid molecule with a calculated pK_a of 2.8 and pK_b of 11. Selected reaction monitoring was chosen because it was highly selective with no interfering peaks for ZMC1 and ABBT in the blank plasma sample (Fig. 2). The ESI interface was used to obtain good sensitivity for protonated ions $[M + H]^+$ of ZMC1 (m/z 235.2) and IS (m/z 231.1). Both demonstrated stable, high-abundance targets and were chosen as precursor ions for MS/MS fragmentation analysis. The most abundant fragments of ZMC1 and IS were m/z 178.2 and 150.1 and were selected as quantitation ions (Fig. 1). The mobile phase gradient elution of acetonitrile and water (0.1% formic acid) provided the best peak shape and sensitivity. The formic acid in the mobile phase increased the ionization of both ZMC1 and internal standard and improved the sensitivity of the assay.

Linearity was not achieved using either acetonitrile–water (80:20, v/v) or acetonitrile deproteinized plasma samples since the peak area decreased significantly at lower concentrations (<100 ng/mL, Fig. 3). This was also observed in plasma matrix using

methanol to deproteinize. Based on the thiosemicarbazone chelator characteristics and an HPLC method reported in the literature (Stariat *et al.*, 2009, 2014), we tested mobile phase A with addition of 2 mM EDTA and found the EDTA precipitated quickly on the ion source cone and reduced the signal. The addition of EDTA (4 μ L, 0.5 M) to plasma (50 μ L) demonstrated a good linearity for ZMC1 standards and with acceptable recovery of >80% (Table 2) for ZMC1 and IS, which also resulted in improvement in peak area, sensitivity and peak shape of ZMC1. Figure 2 shows the LC-MS/MS chromatograms of ZMC1 and IS in human plasma with and without EDTA. The EDTA, supplemented in the samples, was eluted at a retention time around 2 min as detected by the PDA (Photodiode Array detector). It was diverted from the ion source using a divert valve and there can be no ion enhancement effect. The improvement of peak area and peak shape at low concentration of ZMC1 could be hypothesized to be due to EDTA saturation of trace metal ions in the LC system and inhibition of transition ions of ZMC1. The addition of EDTA improved the peak shape, which was also reported by Stariat *et al.* (2014).

LC-MS/MS assay validation

Specificity and linearity—Specificity was evaluated by comparing the selected reaction monitoring chromatogram of blank plasma samples with plasma samples spiked with ZMC1 and IS. No interfering/coeluting peak was detected in the retention times of ZMC1 (5.1 min) and IS (6.2 min) in different batches of plasma (Fig. 2). Calibration standard curves for ZMC1 were plotted using concentrations ranging from 3.9 to 500 ng/mL using weighting factor of 1/concentration. Several calibration standard curves were prepared and the correlation coefficient (r) was 0.997 ($n = 3$).

Precision and accuracy—Table 1 summarizes precision and accuracy results of quality control samples in plasma at three different concentrations. The RSD for both intraday and interday was <10.5% and the bias ranged from -5.4 to 2.1%. The method showed acceptable intra- and inter-assay precision and accuracy according to the US Food and Drug Administration guidelines for bioanalytical method validation. The lower limit of quantitation was 3.9 ng/mL, providing the required quantitation of ZMC1 in preclinical pharmacokinetic studies in mice.

Matrix effects and extraction recovery—Matrix effect causes a compound's response to differ when analyzed in a biological matrix compared with a standard solution, through either ionization suppression or enhancement, compromising the method accuracy. Two common methods are used to evaluate the matrix effects: the post-column infusion method and post-extraction spike method (Matuszewski *et al.*, 2003). In this paper, the post-extraction spike method was used to evaluate the matrix effects. Since there was complete separation of ZMC1 and IS, no relative effect for ZMC1 and IS was observed. Owing to the dilution of the plasma matrix and low volume injection, plasma matrix effects were negligible in this study. As listed in Table 2, the matrix effects on signal were in the range of 99.0–106.6% for the three different ZMC1 QC levels and 92.1% for IS. The absolute recoveries for plasma sample extraction of ZMC1 and IS at the three different QC levels were 92.8 ± 2.2 , 86.1 ± 5.3 , 84.2 ± 12.5 and $93.8 \pm 4.3\%$, respectively.

Stability of ZMC1—Stability of ZMC1 in different solvents (acetonitrile–water, 80:20 v/v, PBS, 0.1 M HCL and 0.1 M NaOH) was investigated at 500 ng/mL at 4 °C, room temperature and 37 °C. ZMC1 exhibited good stability in the solvent acetonitrile and PBS (pH 7.2), which was stable without degradation for 24 h at 4 °C and 37 °C and at room temperature. However, ZMC1 was quickly degraded in acidic buffer due to the hydrolysis of thiosemi-carbazone bond (Fig. 4), which was also observed in thiosemi-carbazone compound of Dp44mT (Stariat *et al.*, 2009). The stability of ZMC1 provides the instruction of the formulation in pre-clinical pharmacokinetics studies. Owing to the acid hydrolysis, this suggests choosing the parenteral route for drug administration (intravenous, intraperitoneal, subcutaneous) or protection of ZMC1 using novel formulation design under gastrointestinal degradation.

The stability test of ZMC1 in plasma (100 and 500 ng/mL) was performed at room temperature and on ice. The percentage of ZMC1 remaining in plasma as a function of storage time is plotted in Fig. 5. ZMC1 in plasma was stable at concentration 500 ng/mL at room temperature for up to 24 h, while >40% of low-concentration ZMC1 (100 ng/mL) was degraded over 24 h, when compared with freshly prepared samples. We assume that the low concentration ZMC1 in plasma has a high potential for hydrolysis owing to the competition between binding with plasma protein and chelating with the metal ion in plasma. The plasma standards and the sample preparation should be kept on ice to avoid ZMC1 degradation in plasma during pharmacokinetic collection procedures. The stability results under various conditions throughout the validation process are shown in Table 3. The experiments revealed that ZMC1 is stable when the plasma samples are stored at –80 °C for 1 month, at 4 °C for 2 h and after three cycles of freeze–thawing, and for 24 h kept in the autosampler under experimental conditions. All the stability results were within the assay variability limits during the entire experiments.

***In vitro* plasma protein binding of ZMC1**

The plasma–protein binding of drugs is an important pharmacokinetic evaluation for drug development. We investigated the ZMC1 plasma–protein binding by ultrafiltration and ultracentrifugation method. In preliminary experiments, three different concentrations of ZMC1 (500, 31.25 and 3.9 ng/mL) were chosen to evaluate the plasma–protein binding characteristics. The concentration of free ZMC1 in ultrafiltrate or ultracentrifugation supernatant was below the detection limit at concentrations of 31.25 and 3.9 ng/mL. Therefore, the plasma–protein binding of ZMC1 was performed only at the concentration of 500 ng/mL. As shown in Table 4, ZMC1 was highly bound to plasma protein (98.4%). The binding experiments of ZMC1 in solutions of HSA (4%) and human AAG (0.1%) indicated that HSA could be the major carrier for ZMC1 in plasma.

Conclusions

We have developed a sensitive, and accurate LC/MS/MS assay to quantify ZMC1 in plasma. This validated assay with good accuracy and precision using 50 µL of plasma for ZMC1 determination with the lower limit of quantification of 3.9 ng/mL is superior to the HPLC–UV assay. The sample preparation with the addition of EDTA increased the sensitivity and

achieved a good linearity of the standards for ZMC1 in plasma matrix. Preliminary stability studies demonstrated good stability of ZMC1 in neutral buffer. The LC/MS/MS assay provides a suitable method for further investigation of the ZMC1 metabolites in preclinical and clinical pharmacokinetics studies.

Acknowledgments

The authors gratefully acknowledge the financial support provided by the Cancer Institute of New Jersey, New Brunswick, NJ and National Institutes of Health grants P30ES005022, NCI Cancer Center Support Grant P30CA072720, NCI K08CA172676-02, Sidney Kimmel Foundation for Cancer Research and Pancreatic Cancer Action Network to D.C..

Abbreviations used

AAG	human α_1 -acid glycoprotein
ABBT	2-amino-6-bromobenzothiazole
HSA	human serum albumin

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Figure 1. Chemical structure of ZMC1 (a) and internal standard 2-amino-6-bromobenzothiazole (b). Mass spectrum of ZMC1 product ion of $[M + H]^+$ at m/z 235.3 and internal standard product ion of $[M + H]^+$ at m/z 231.1.

Figure 2.

Representative LC-MS/MS chromatograms of ZMC1 and IS in human plasma. (a) Blank plasma matrix; (b, c) plasma standard 3.9 ng/mL, 500 ng/mL with EDTA; (d, e) plasma standard 3.9 ng/mL, 500 ng/mL without addition of EDTA. Red line, ZMC1; black line, IS.

Figure 3.

Effect of EDTA on the calibration curves of ZMC1 in solvent (acetonitrile–water, 80:20, v/v) and plasma with weighting factor of $1/x$. (diamond) $y = -0.00222 + 0.00409x$, $R^2 = 0.9988$; (triangle) $y = 0.00161 + 0.00404x$, $R^2 = 0.9995$; (square) $y = -0.0133 + 0.00237x$, $R^2 = 0.9608$; (cycle) $y = 0.00751 + 0.00175x$, $R^2 = 0.9656$.

Figure 4. Stability of ZMC1 in acetonitrile–water (80:20, v/v), PBS (pH = 7.2), 0.1 M HCl and 0.1 M NaOH for 24 h at room temperature, 4°C and 37 °C.

Figure 5.
Stability of ZMC1 (100, 500 ng/mL) in plasma at room temperature and on ice.

Table 1

Precision and accuracy of ZMC1 LC/MS/MS assay in human plasma

ZMC1 nominal concentration (ng/mL)	Intraday (<i>n</i> = 3)			Interday (<i>n</i> = 4)		
	Measured (ng/mL, mean ± SD)	RSD (%)	Bias (%)	Measured (ng/mL, mean ± SD)	RSD (%)	Bias (%)
500.0	510.3 ± 30.2	5.9	2.1	495.1 ± 10.5	2.1	1.0
31.3	29.6 ± 1.1	3.8	-5.4	31.4 ± 3.3	10.5	0.6
3.9	3.9 ± 0.12	3.1	-0.5	3.9 ± 0.05	1.3	-0.5

Table 2Recovery and matrix effect of ZMC1 and IS in human plasma ($n = 3$)

Components	Concentration (ng/mL)	Recovery, mean $\pm$ SD (%)	Matrix effect, mean $\pm$ SD (%)
ZMC1	500.0	92.8 $\pm$ 2.2	99.0 $\pm$ 2.2
	31.3	86.1 $\pm$ 5.3	106.6 $\pm$ 1.8
	3.9	84.2 $\pm$ 12.5	106.1 $\pm$ 6.8
IS	500.0	93.8 $\pm$ 4.3	92.1 $\pm$ 1.8

Table 3Stability of ZMC1 in human plasma under different storage conditions ($n = 3$)

Storage conditions	Concentration (ng/mL)	Measured concentration (ng/mL)	RSD (%)	Bias (%)
Short-term (2 h on ice)	500.0	493.9	3.9	-1.3
	31.3	29.0	0.7	-7.0
	3.9	3.7	0.4	-0.4
Long term (30 days at -80 °C)	500.0	516.4	4.8	3.2
	31.3	30.3	11.2	-2.9
	3.9	3.9	1.9	-0.01
Three freeze– thaw cycles	500.0	553.1	553.1	10.6
	31.3	29.0	28.0	-10.4
	3.9	4.1	4.1	4.8
Autosampler for 24 h	500.0	550.7	1.6	10.0
	31.3	35.4	0.7	13.0
	3.9	4.1	2.3	4.0

Table 4

In vitro ZMC1 binding to human plasma and plasma protein solutions determined by ultrafiltration and ultracentrifugation (n = 3)

Matrix	Percentage of bound drug by	
	ultrafiltration	ultracentrifugation
Plasma	98.8	97.5
Human serum albumin (4%)	98.4	N/D
Human α_1 -acid glycoprotein (0.1%)	34.9	N/D