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Determination of Triapine, A Ribonucleotide Reductase Inhibitor, In Human Plasma by Liquid Chromatography Tandem Mass Spectrometry

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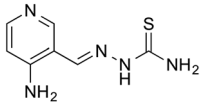
Introduction

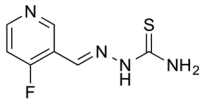
Ribonucleotide reductase (RNR) is an essential enzyme for cell division as well as tumor growth (Finch *et al*., 1999), which not only catalyzes the conserved reduction of ribonucleotides to deoxyribonucleotides, but also regulates the total rate of DNA synthesis to maintain a constant ratio of DNA to cell mass during cell proliferation and DNA repair (Jordan and Reichard, 1998; Shao *et al*., 2006; Xu *et al*., 2008). There are two RNR isoforms in human (two dimers of M1–M2 subunits, or two dimers of M1–M2b subunits), and the catalytic function of the enzyme relies on the essential iron-tyrosyl radical center in M2 or M2b subunits (Jordan and Reichard, 1998). Hence, it is a prime target for the development of chemotherapeutic agents.

Triapine (3-aminopyridine-2-carboxaldehyde thiosemicarbazone, or 3-AP; Fig. 1A) is a metal-ion chelator and an experimental anticancer agent, which can disrupt the catalytic iron-tyrosyl radical center in M2 or M2b subunit of RNR via the formation of triapine–Fe(III) complex (Kowol *et al*., 2009). Compared with hydroxyurea (HU), the only RNR-inhibitor drug currently used in chemotherapy, triapine is 1000-fold more potent for inhibition of RNR activity (Finch *et al*., 1999). Studies have shown that triapine has a broader-spectrum antitumor activity and can significantly decrease RNR activity in a variety of cancer cell lines including leukemia, nonsmall-cell lung cancer, renal cancer and melanoma, and enhances radiation-mediated cytotoxicity in cervical and colon cancers (Alvero *et al*., 2006; Finch *et al*., 1999, 2000; Giles *et al*., 2003; Kuo *et al*., 2003; Li *et al*., 2001). These promising pre-clinical studies have prompted a large number of clinical trials. To date, there have been 36 clinical trials of triapine worldwide for various cancers at various stages of studies (http://www.clinicaltrials.gov/ct2/results?term=triapine&Search=Search, accessed 14 December 2014). At the Case Comprehensive Cancer Center, the triapine studies have been focused on the investigation of sensitizing potential of triapine on radiation therapy of cervical and vaginal cancers (Kunos *et al*., 2009, 2010a, 2010b, 2013).

Despite the therapeutic significance of triapine, there is no validated analytical method published for quantitation of triapine in human biological samples. Several LC-UV assays have been described for the measurement of triapine in clinical studies (Mortazavi *et al*., 2013; Murren *et al*., 2003; Yee *et al*., 2006); however, when adopted, they often produced irreproducible results and were hindered by poor selectivity and inadequate limits of quantitation. Our recent study revealed that factors including solution pH, metal ions, other competing chelators and buffer composition can affect the reproducibility of an LC-UV method by influencing triapine complexation reaction, and the



(A) Triapine (3-AP)



(B) NSC 266749 (IS)

Figure 1. The chemical structures of triapine and NSC 266749 (IS).

optimization of these factors is critical in the chromatography method development (Feng *et al*., 2014).

In this work, we have developed and validated a reliable and selective LC-MS/MS method based on our recent findings (Feng *et al*., 2014) with adequate lower limit of quantitation (LLOQ) for determination of triapine in human plasma samples. Plasma samples were prepared by direct protein precipitation with acetonitrile. The method developed has been applied to the measurement of triapine in patient samples obtained from a phase I clinical trial.

Experimental

Chemicals and materials

Triapine was provided by Vion Pharmaceuticals (New Haven, CT, USA) and used as the chemical standard for this work. 2 [(3 Fluoro 2 pyridinyl)methylene] hydrazinecarbothioamide (NSC 266749 or CAS 31181 41 6) was obtained from the Developmental Therapeutics Program of the National Cancer Institute at the National Institutes of Health (Bethesda, MD, USA) and used as the internal standard (IS). HPLC grade methanol, ethylenediaminetetraacetic acid disodium salt (EDTA), and ammonium hydroxide were purchased from Sigma Aldrich (St Louis, MO, USA). Ammonium bicarbonate was from EMD Chemicals (Darmstadt, Germany). Deionized water was obtained from a Barnstead Model 7148 Nanopure® ultrapure water system (Thermo Scientific, Asheville, NC, USA). Six lots of human blank plasma (EDTA treated), six lots of human blank sera, and pooled human blank plasma were purchased from Innovative Research (Novi, MI, USA).

Preparation of stock and standard solutions

The stock solutions of triapine (1.00 mg/mL) and NSC 266749 (1.00 mg/mL) were prepared individually by dissolving appropriate amount of each chemical in a known volume of methanol, and were kept at −20°C before use. The dilution solution which was prepared for dilution of the stock solutions to working standard solutions contained 25.0% acetonitrile and 75.0% ammonium bicarbonate EDTA (10.0 mM NH_4HCO_3 and 1.00 mM EDTA, pH 8.50) buffer (v/v). The working solutions of triapine (1.00 μg/mL) and NSC 266749 (1.00 μg/mL) were freshly prepared by serial dilution of each stock solution with the dilution solution.

Triapine standard solutions (5.00, 10.0, 15.0, 20.0, 50.0, 100, 150, 200, 500, 800 and 1.00×10^3 ng/mL) and NSC 266749 internal standard solution (100 ng/mL) were prepared individually by serial dilution of the working solutions of triapine (1.00 μg/mL) and NSC 266749 (1.00 μg/mL) with the dilution solution. These standard solutions were used for the preparation of triapine plasma calibrators and quality controls (QCs).

Preparation of triapine plasma calibrators, QCs and patients' samples

Triapine plasma calibrators (0.250, 0.500, 1.00, 2.50, 5.00, 10.0, 25.0 and 50.0 ng/mL) and plasma quality controls (i.e. low , medium , high and dilution QCs 0.750, 7.50, 40.0 and 400 ng/mL) were prepared individually by mixing 200 μL of pooled blank human plasma and 10.0 μL of the corresponding triapine standard solution, which was 20 times the calibrator concentration. Patient plasma samples were prepared by mixing 200 μL of each patient's plasma with 10.0 μL of the dilution solution to match the sample matrix with those of the plasma calibrators and QCs.

Single blank plasma (or triapine plasma zero calibrator) was prepared by mixing 200 μL of pooled blank human plasma with 10.0 μL of the dilution solution. Double blank plasma (containing neither triapine nor the IS) was prepared by mixing 200 μL of pooled blank human plasma with 20.0 μL of the dilution solution.

Prior to sample deproteinization, 10.0 μL of the IS solution (100 ng/mL) was added to each of the above plasma samples (e.g. calibrators including zero calibrator, QCs and patient samples) except double blank plasma and mixed well.

Deproteinization of plasma samples

Each plasma sample was mixed with 880 μL of acetonitrile (at a ratio of 4 to 1) and vortexed for 30 s. Following by centrifugation at 15,000 × g for 10 min, the supernatant was pipetted into a 1.5 mL microcentrifuge tube and dried in a TurboVap® LV evaporator (Caliper Life Sciences, Hopkinton, MA, USA) at 30°C under nitrogen gas. The resulting residue was reconstituted in 110 μL of the dilution solution for LC MS/MS analysis.

Instrumentation

The LC MS/MS instrumentation system used consisted of a Shimadzu SIL 20AC autosampler (Shimadzu, Columbia, MD, USA), a Shimadzu LC 20AD HPLC unit with a Waters Xbridge Shield RP_{18} column (3.5 μm; 2.1 × 50 mm) and a mobile phase containing methanol and 10.0 mM ammonium bicarbonate (pH 8.50) at a ratio of 25:75 (v/v) and an AB Sciex API 3200 turbo ionspray® triple quadrupole tandem mass spectrometer (AB Sciex, Foster City, CA, USA). The system was controlled by AB Sciex Analyst® (version 1.5.1) software.

The API 3200 tandem mass spectrometer was operated under the positive turbo ionspray ionization mode and was tuned by a mixture of 500 ng/mL tiapine and 500 ng/mL the IS in 50% methanol and 50% ammonium bicarbonate (10.0 mM, pH 8.50) for both compound dependent and source dependent parameters. The multiple reaction monitoring (MRM) data were acquired with the following mass transitions: *m/z* 196 > 121 for triapine, and *m/z* 199 > 124 for the IS. The optimized instrument settings were as follows: curtain gas at 40; collision assisted dissociation gas at 8; ionization voltage at 4500 V; source temperature at 700; sheath gas at 50; desolvation gas at 40; desolvation potential at 40; entrance potential at 4; collision energy at 23; collision cell exit potential at 2; and resolution at unit.

The separation of triapine and the IS was accomplished by isocratic elution with the mobile phase at a flow rate of 0.300 mL/min. Prior to initial sample analysis, the column was equilibrated with the mobile phase at the flow rate for at least 30 min. During each run, 10.0 μL of reconstituted sample was injected into the system by the autosampler set at 4.0°C. The two position switch valve on the API 3200 tandem mass spectrometer was programmed to switch to the waste for the first 1.2 min and then switch to the mass spectrometer. Quantitation of triapine was carried out by the MRM mode of the tandem mass spectrometer, and the total instrument run time for each sample analysis was 4 min.

Method validation

The LC MS/MS method developed was validated in human plasma according to the US Food and Drug Administration (2001) guidance for industry on bioanalytical method validation and the white paper of the 2006 bioanalytical method validation workshop (Bansal and DeStefano,

2007) in terms of selectivity, LLOQ, recovery, matrix effect, linear response range, accuracy and precision, as well as stability for both short term sample processing and long term sample storage.

Selectivity and LLOQ

The selectivity of this method was evaluated by observing any interfernts at the same retention times and mass transitions of the analyte and the IS in six individual blank plasma and pooled blank plasma matrices, as well as in the pre dosed plasma from patients. The LLOQ of the method was defined as the lowest concentration of triapine plasma calibrator of the calibration curve with accuracy and precision $\leq \pm 20$ and 20%, which was validated in six individual plasma, and pooled plasma matrices.

Matrix factor and recovery

The absolute matrix factor (MF) of triapine (or the IS) was determined by the mean peak area of triapine (or the IS) at a specified concentration in the deproteinized plasma matrix over that of triapine (or the IS) at the concentration in the mobile phase. The IS normalized MF was determined by the absolute MF of triapine over that of the IS. For this study, triapine QCs at two concentrations (0.750 and 40.0 ng/mL) with a fixed concentration of the IS (5.00 ng/mL) were prepared in six individual deproteinized plasma matrices and in the mobile phase.

The absolute recovery of triapine (or the IS) was determined by the mean peak area of triapine (or the IS) at a specific concentration in plasma matrix over the mean peak area of triapine (or the IS) at the concentration in the deproteinized plasma matrix multiplied by 100%. The IS normalized recovery was determined by the absolute recovery of triapine over that of the IS multiplying by 100%. For this study, triapine QCs at three concentrations (0.750, 7.50 and 40.0 ng/mL) with a fixed concentration of the IS (5.00 ng/mL) were prepared in the pooled human plasma and the deproteinized pooled human plasma.

Calibration curve

The triapine calibration curve in human plasma was established using double blank (plasma matrix with neither triapine nor the IS), single blank (plasma matrix with only the IS) and eight nonzero plasma calibrators at the concentrations 0.250, 0.500, 1.00, 2.50, 5.00, 10.0, 25.0 and 50.0 ng/mL with the IS at the concentration 5.00 ng/mL. The peak area ratios of triapine to the IS (y) were plotted against triapine calibrator concentrations (x) with $1/x$ weighting.

Accuracy, precision and dilution study

Accuracy was expressed as percentage relative error (RE) and precision as the coefficient of variation (CV). In this work, the inter assay precision and accuracy were assessed by five parallel analyses of five identical QC samples at each of four QC concentrations (0.750, 7.50, 40.0 and 400 ng/mL). The intra assay precision and accuracy were assessed by five replicate analyses of each QC samples. The above studies also included the dilution study where the dilution QCs (400 ng/mL) were prepared and analyzed after 10 fold dilution by the pooled human blank plasma.

Stability study

The stability of triapine was investigated using stock and working solutions (1.00 mg/mL and 1.00 μg/mL) and plasma low and high QCs (0.750 and 40.0 ng/mL), and the stability of NSC 266749 was also determined separately using NSC 266749 standard solution (100 ng/mL) and plasma sample containing 5.00 ng/mL of NSC 266749. In the NSC 266749 stability studies, triapine was used as the IS.

Stabilities of triapine stock and working solutions, triapine plasma QCs, NSC 266749 standard solution and plasma samples were assessed during short term (6 and 24 h) storage, standing on the bench top at 23°C and in the autosampler at 4°C (post preparative); during three freeze thaw cycles (where the samples were frozen at −20°C for at least 24 h and thawed at room temperature, 23°C, unassisted); and during long term storage (30 days) at −20°C. The stabilities of triapine and NSC 266749 were determined by comparing the mean peak area ratios of analyte to IS in the test sample with those of freshly prepared samples, and expressed as percentages. For this study, all experiments were done in five replicates.

Method application

The LC MS/MS method developed was tested for the measurement of triapine in patient plasma samples collected from a previous phase I

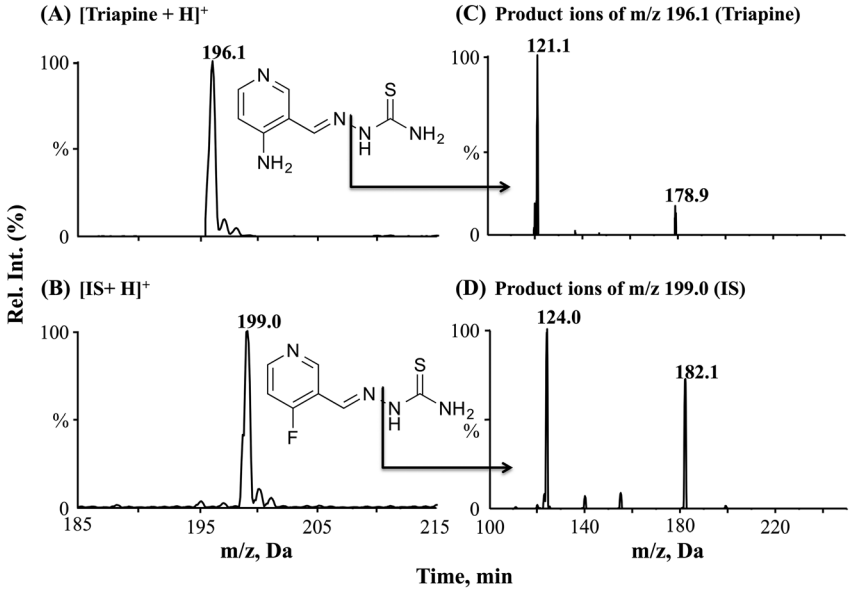


Figure 2. The mass spectra of triapine and the IS. The experimental conditions were described in the 'Instrumentation' section under the 'Experimental'.

clinical trial of pelvic radiation, weekly cisplatin and triapine for locally advanced cervical cancer (Kunos *et al*., 2010a, 2010b), where patients were given triapine by a 2 h intravenous (i.v.) infusion at the dose of 25 mg/m^2 three times a week for 5 weeks. Blood samples were collected on days 1 and 10 in heparinized tubes at the following time points: 0 (pre dose), 2, 4, 6 and 24 h after the start of 2 h i.v. infusion. Plasma samples were harvested by a refrigerated centrifuge, and then stored at −80°C before analysis.

In the analysis, patient samples together with 10 calibrators (i.e. double and single blank, and eight nonzero) and a set of QCs at low, medium and high concentrations (i.e. 0.750, 7.50 and 40.0 ng/mL) were first prepared, deproteinized and processed according to the procedures described in the Experimental section, then analyzed by the validated method. The patient samples having concentrations beyond the upper limit of quantitation were re run with the dilution QC after 1:10 dilution with the pooled human blank plasma.

Results and discussion

Mass spectrometric detection

In this work, the optimization of triapine and the IS signal responses was done using the 'auto-tune' function of AB Sciex Analyst software (version 1.5.1). Since triapine and the IS more easily give their protonated than deprotonated forms in electrospray ionization (ESI), the positive ESI was used for triapine identification and quantification. As shown in Fig. 2(A and C), the predominated tirapine and IS molecular ions were at *m/z* 196 and *m/z* 199, respectively. These protonated molecules could further be broken down into product ions by collision with nitrogen gas and produced the predominant product ions at *m/z* 121 and *m/z* 124 (Fig. 2B and D). Therefore, the mass transitions at *m/z* 196 > 121 for triapine and *m/z* 199 > 124 for the IS were chosen for quantitation of triapine by MRM mode.

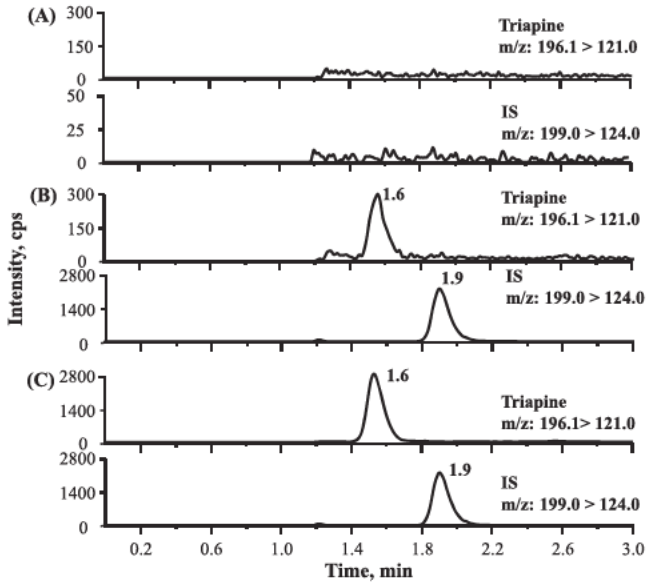


Figure 3. The representative multiple reaction monitoring chromatograms. (A) Double blank plasma; (B) 0.250 ng/mL triapine plasma calibrator (with 5.00 ng/mL IS); and (C) 2.50 ng/mL triapine plasma calibrator (with 5.00 ng/mL IS).

Liquid chromatographic separation

In this work, separation of triapine and the IS was tested on several analytical columns, including a Waters X-Terra RP 18 (2.1 × 150 mm, 5 μm particle size), a Waters X-Bridge RP_{18} (2.0 × 50 mm, 5 μm particle size), a Waters X-Bridge AQ (2.0 × 50 mm, 5 μm particle size) and a Waters X-Bridge Phenyl (2.0 × 50 mm, 5 μm particle size), with a mobile phase containing 25% organic solvent (either methanol or acetonitrile) and 75% ammonium bicarbonate (10.0 mM, pH 8.50) at a flow rate of 0.300 mL/min. Although reasonable retention time and baseline resolution were achieved on all columns tested with either organic modifier, the Waters X-Bridge RP_{18} column and methanol solvent were chosen for the latter method development because they gave greater separation efficiency and detection sensitivity for triapine and the IS. The optimal separation of triapine and the IS was achieved on a Waters X-Bridge RP_{18} column at 1.6 and 1.9 min using a mobile phase containing 25% methanol and 75% ammonium bicarbonate (10.0 mM, pH 8.50, v/v; Fig. 3).

EDTA

Triapine is a metal-ion chelator, and can easily react with metal ions in an aqueous solution (Feng *et al*., 2014). Our experimental data indicated that even the trace metal ions in the presence of the deionized water (18.2 MΩ cm) obtained from a Barnstead Nanopure Water System could affect the reproducibility of analytical signal of triapine by LC-MS/MS, unless a stronger metal-ion chelator (e.g. EDTA) was present in the same solution

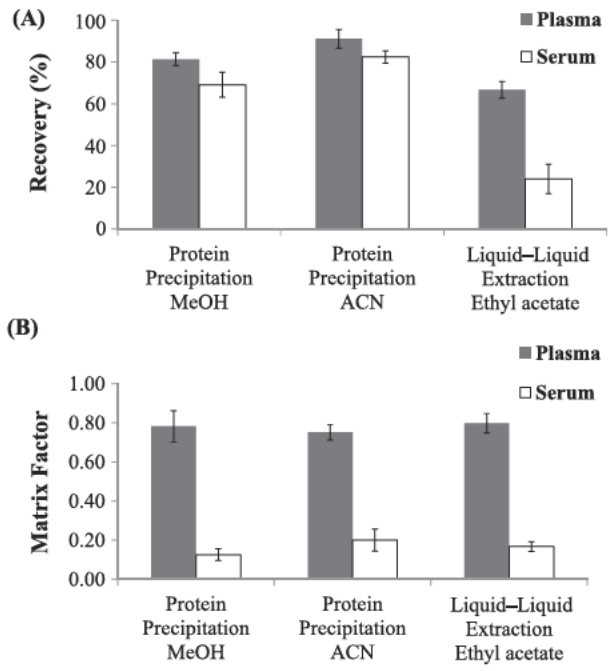


Figure 4. Comparison of matrix effect and recovery of triapine from human plasma vs serum by different sample preparation protocols. Each column represents the mean ± SD from six lots of each sample matrix. Three sample preparation protocols include: (1) protein precipitation by methanol (1:4; v/v); (2) acetonitrile (1:4; v/v); and (3) liquid liquid extraction by acetyl acetate (1:2; v/v). The triapine concentration was 7.50 ng/mL in each plasma and serum matrix.

to preserve triapine from complexation. In this work, a dilution solution containing 25.0% acetonitrile and 75.0% ammonium bicarbonate–EDTA buffer (10.0 mM NH_4HCO_3 and 1.00 mM EDTA, pH 8.50) was used for preparation of triapine working solution from its stock in methanol, as well as triapine standards from the working solution. It is also worth noting that triapine working and standard solutions prepared in Ultrapure HPLC-grade bottled water from Fisher Scientific (Fair Lawn, NJ, USA) could also give reproducible analytical signal if it was not contaminated by metal ions.

Plasma samples vs serum samples

Because EDTA can preserve triapine from complexation with metal ions, a comparison study was conducted on the absolute recovery and matrix effect of triapine from EDTA-treated human plasma and serum samples using two types of preparation methods, protein precipitation and liquid–liquid extraction. In this work, triapine plasma and serum samples (at 7.50 ng/mL) were prepared by spiking triapine standard solution in six lots of human blank plasma and six lots of human blank serum. These samples were prepared by protein precipitation with acetonitrile and methanol, as well as liquid–liquid extraction with acetyl acetate. As shown in Fig. 4, the absolute recoveries of triapine were consistently higher from EDTA-treated plasma matrices (67–91%) than those from serum matrices (24–69%) by both types of sample preparation methods, and the absolute matrix factors were much closer to unity for plasma samples (0.750–0.800) than those of serum samples (0.120–0.200). Furthermore, protein precipitation with 4 volumes of acetonitrile produced greater recovery for triapine and comparable matrix factor among the sample preparation methods. Therefore, EDTA-treated plasma samples and protein precipitation by acetonitrile were used for the method development and sample preparation in the subsequent work. For clinical study of triapine, EDTA-treated tubes are highly recommended for blood collection.

Table 1. Accuracy and precision of triapine at lower limit of quantitation (LLOQ) in six lots of human plasmas (n = 5)

Plasma matrix	Nominal [triapine] (ng/mL)	Measured [triapine] (ng/mL)	SD (ng/mL)	Precision (CV, %)[a]	Accuracy (RE, %)[b]
Lot 1	0.250	0.225	0.004	2	−10
Lot 2	0.250	0.26	0.02	8	4
Lot 3	0.250	0.26	0.01	4	4
Lot 4	0.250	0.248	0.007	3	−1
Lot 5	0.250	0.261	0.007	3	4
Lot 6	0.250	0.250	0.008	3	0
Mean (6 lots)	0.250	0.250	0.004	2	0

[a]CV = (standard deviation/mean) × 100%.
[b]RE = [(measured − nominal)/nominal] × 100%.

Selectivity and LLOQ

The LC-MS/MS method developed was highly selective. As illustrated in Fig. 3(A), there were no detectable interferents observed at the retention times and mass transitions of triapine and the IS from six individual plasma and the pooled plasma matrices, as well as the pre-dosed plasma from patients in a phase I clinical trial (Kunos *et al*., 2010a, 2010b).

The LLOQ of the method for quantitation of tripaine in human plasma was 0.250 ng/mL, which had signal-to-noise ratio of 23.5 Fig. 3B). Table 1 summarized the accuracy and precision of the

Table 2. Matrix effect of triapine in six individual lots of blank human plasma (n = 5)

Plasma matrix	[Triapine] (ng/mL)	$PA_{Triapine}$[a] in extracted plasma ± SD (×10^3)	$PA_{Triapine}$ in mobile phase ± SD (×10^3)	$MF_{Triapine}$[b] ± SD[c]	PA_{IS}[d] in extracted plasma ± SD (×10^3)	PA_{IS} in mobile phase ± SD (×10^3)	MF_{IS}[e] ± SD	IS normalized MF[f] ± SD
Lot 1	0.750	4.9 ± 0.4	6.4 ± 0.2	0.77 ± 0.07	22 ± 2	27.6 ± 0.5	0.80 ± 0.07	1.0 ± 0.1
	40.0	265 ± 5	362 ± 2	0.73 ± 0.01	21.8 ± 0.8	28.3 ± 0.3	0.77 ± 0.03	0.95 ± 0.04
Lot 2	0.750	5.0 ± 0.3	6.4 ± 0.2	0.78 ± 0.05	22 ± 1	27.6 ± 0.5	0.80 ± 0.04	0.98 ± 0.08
	40.0	$2.7 \times 10^2 \pm 1 \times 10^1$	362 ± 2	0.75 ± 0.03	21.5 ± 0.7	28.3 ± 0.3	0.76 ± 0.03	0.99 ± 0.06
Lot 3	0.750	4.5 ± 0.2	6.4 ± 0.2	0.70 ± 0.04	21 ± 1	27.6 ± 0.5	0.76 ± 0.04	0.92 ± 0.07
	40.0	243 ± 6	362 ± 2	0.67 ± 0.02	19.9 ± 0.5	28.3 ± 0.3	0.70 ± 0.02	0.96 ± 0.04
Lot 4	0.750	4.4 ± 0.3	6.4 ± 0.2	0.69 ± 0.05	20 ± 2	27.6 ± 0.5	0.72 ± 0.07	1.0 ± 0.1
	40.0	206 ± 8	362 ± 2	0.57 ± 0.02	18 ± 1	28.3 ± 0.3	0.64 ± 0.04	0.89 ± 0.06
Lot 5	0.750	4.7 ± 0.2	6.4 ± 0.2	0.73 ± 0.04	19 ± 1	27.6 ± 0.5	0.69 ± 0.04	1.06 ± 0.08
	40.0	$2.4 \times 10^2 \pm 3 \times 10^1$	362 ± 2	0.66 ± 0.08	19 ± 2	28.3 ± 0.3	0.67 ± 0.07	1.0 ± 0.1
Lot 6	0.750	4.5 ± 0.2	6.4 ± 0.2	0.70 ± 0.04	19.6 ± 0.9	27.6 ± 0.5	0.71 ± 0.04	0.99 ± 0.08
	40.0	$2.3 \times 10^2 \pm 1 \times 10^1$	362 ± 2	0.64 ± 0.03	19.6 ± 0.2	28.3 ± 0.3	0.69 ± 0.01	0.93 ± 0.05

[a]$PA_{Triapine}$ = mean peak area of triapine.
[b]$MF_{Triapine}$ = ($PA_{Triapine}$ in extracted plasma matrix)/($PA_{Triapine}$ in mobile phase).
[c]$y\ (\pm s_y) = [a(\pm s_a)/b(\pm s_b)]$, $s_y = y \times \sqrt{[(s_a/a)^2 + (s_b/b)^2]}$.
[d]PA_{IS} = mean peak area of IS.
[e]MF_{IS} = (PA_{IS} in extracted plasma matrix)/(PA_{IS} in mobile phase).
[f]IS normalized MF = $MF_{Triapine}/MF_{IS}$.

Table 3. Recovery of triapine in pooled human plasma (n = 5)

[Triapine] (ng/mL)	$PA_{Triapine}$[a] in plasma ± SD ($\times10^3$)	$PA_{Triapine}$ in extracted plasma ± SD ($\times10^3$)	$Recovery_{Triapine}$[b] ± SD (%)	PA_{IS}[c] in plasma ± SD ($\times10^3$)	PA_{IS} in extracted plasma ± SD ($\times10^3$)	$Recovery_{IS}$[d] ± SD (%)	IS normalized recovery[e] ± SD (%)
0.750	3.2 ± 0.2	3.6 ± 0.1	89 ± 6	13.6 ± 0.7	15.5 ± 0.4	88 ± 5	101 ± 9
7.50	37.8 ± 0.8	41.5 ± 0.6	91 ± 2	13.4 ± 0.3	15.3 ± 0.4	88 ± 3	103 ± 4
40.0	214 ± 5	230 ± 5	93 ± 3	13.9 ± 0.3	15.6 ± 0.2	89 ± 2	104 ± 4

[a]$PA_{Triapine}$ = mean peak area of triapine.
[b]$Recovery_{Triapine}$ = [($PA_{Triapine}$ in plasma matrix)/($PA_{Triapine}$ in extracted plasma matrix)] × 100%.
[c]PA_{IS} = mean peak area of IS.
[d]$Recovery_{IS}$ = [(PA_{IS} in plasma matrix)/(PA_{IS} in extracted plasma matrix)] × 100%.
[e]IS normalized recovery = [($Recovery_{Triapine}$)/($Recovery_{IS}$)] × 100%.

method at LLOQ. By five replicate measurements of each plasma matrix from six lots, the accuracy and precision were ≤±10 and ≤8%, respectively. These values were lower than those of the industry limits recommended by US Food and Drug Administration (≤±20 and ≤20%), which implied that the actual LLOQ of the method could be <0.250 ng/mL if it was validated when needed.

Matrix effect and recovery

Matrix effect was assessed by the MF using six independent lots of human plasmas. As shown in Table 2, the absolute MFs of triapine and the IS were 0.57–0.78 and 0.64–0.80, respectively, and the IS normalized MFs were near unity (0.92–1.06). Hence, the matrix effect of human plasma on the analytical signals could be effectively corrected by the use of the IS.

The recovery data of triapine in pooled human plasma is shown in Table 3. The absolute recoveries of triapine and the IS were 89–93 and 88–89%, respectively, and the IS normalized recovery was 101–104%. These results indicated that the protein precipitation procedure was sufficient to recover the analyte and the IS from human plasma.

Linearity

The linear regression equation obtained from six batches over 3 days was $y = 0.373(\pm0.012)x - 0.00107(\pm0.00034)$ over the range 0.250–50.0 ng/mL with individual correlation coefficients >0.999. The accuracy and precision of individual plasma calibrators as summarized in Table 4 were ≤±6 and ≤8%, respectively.

Table 4. Accuracy and precision of plasma calibrators over three different days (n = 6)

Sample matrix	Nominal [triapine] (ng/mL)	Measured [triapine] (ng/mL)	SD (ng/mL)	Precision (CV, %)	Accuracy (RE, %)
Pooled human plasma	0.250	0.25	0.01	4	0
	0.500	0.50	0.03	6	0
	1.00	0.98	0.08	8	−2
	2.50	2.5	0.1	4	1
	5.00	4.9	0.4	8	−2
	10.0	9.8	0.5	5	−2
	25.0	24	2	8	−4
	50.0	53	2	4	6

Accuracy, precision and dilution integrity

As shown in Table 5, the accuracy and precision were ≤±8 and ≤5% for the intra-assay study and ≤±7 and ≤4% for the inter-assay study, indicating the method developed was accurate and precise. In this study, dilution QC (400 ng/mL) was included because some of the patient plasma samples had triapine concentrations beyond the upper limit of quantitation (50.0 ng/mL) of the method. The accuracy and precision data of the dilution QC (≤±2 and ≤0.5% for the intra-assay study and ≤±7 and ≤1% for the inter-assay study) indicated that the integrity of plasma sample could be preserved after sample dilution.

Table 5. Intra- and inter-run accuracy and precision for triapine in pooled human plasma

Nominal [Triapine] (ng/mL)	Measured [Triapine] (ng/mL)	SD (ng/mL)	Precision (CV, %)	Accuracy (RE, %)
Intra-run[a] *(n = 5 replicates)*				
0.750	0.74	0.02	3	−1
7.50	8.1	0.1	1	8
40.0	40	2	5	−1
400[c]	406	2	0.5	2
Inter-run[b] *(n = 5 parallels)*				
0.750	0.72	0.01	1	−4
7.50	7.0	0.3	4	−7
40.0	38.2	0.9	2	−4
400[c]	429	3	1	7

[a]Each datum point calculated by five replicate measurements of each quality control (QC) sample.
[b]Each datum point calculated by five parallel measurements from five identical QCs.
[c]Dilution QC was measured by 10× dilution with blank pooled human plasma.

Table 6. Stabilities of triapine and NSC 266749 under various conditions (n = 5)[a]

Test conditions	Temperature (°C)		Recovery ± SD (%)		
			6 h	24 h	
		Triapine			
Bench-top	23	Stock solution [b] (1.00×10^6 ng/mL)	101 ± 1	100 ± 4	
		Working solution [b] (1.00×10^3 ng/mL)	106 ± 3	100 ± 5	
		Plasma low QC [c]	88 ± 2	68 ± 3	
		Plasma high QC [c]	90 ± 2	61 ± 5	
Autosampler	4	Plasma low QC	96 ± 1	94 ± 8	
		Plasma high QC	87 ± 1	83 ± 5	
Three freeze–thaw cycles	−20 to 23	Plasma low QC			96 ± 2
		Plasma high QC			98 ± 5
Long-term (30 days)	−20	Plasma low QC			86 ± 6
		Plasma high QC			91 ± 5
		NSC 266749[b]			
Bench-top	23	Solution (100 ng/mL)	105 ± 2	100 ± 6	
		Plasma (5.00 ng/mL)	89 ± 5	84 ± 5	
Autosampler	4	Plasma (5.00 ng/mL)	87 ± 3	93 ± 5	
Three freeze–thaw cycles	−20 to 23	Plasma (5.00 ng/mL)			92 ± 4
Long-term (30 days)	−20	Plasma (5.00 ng/mL)			88 ± 7

[a]The measurement of triapine used NSC 266749 (5.00 ng/mL) as IS whereas the measurement of NSC 266749 used triapine (5.00 ng/mL) as IS.
[b]The concentrations of stock solution and working solution were measured by serial dilution to 40.0 ng/mL with the dilution solution as described in the Experimental section.
[c]The concentrations of plasma low and high QCs were 0.750 and 40.0 ng/mL, respectively.

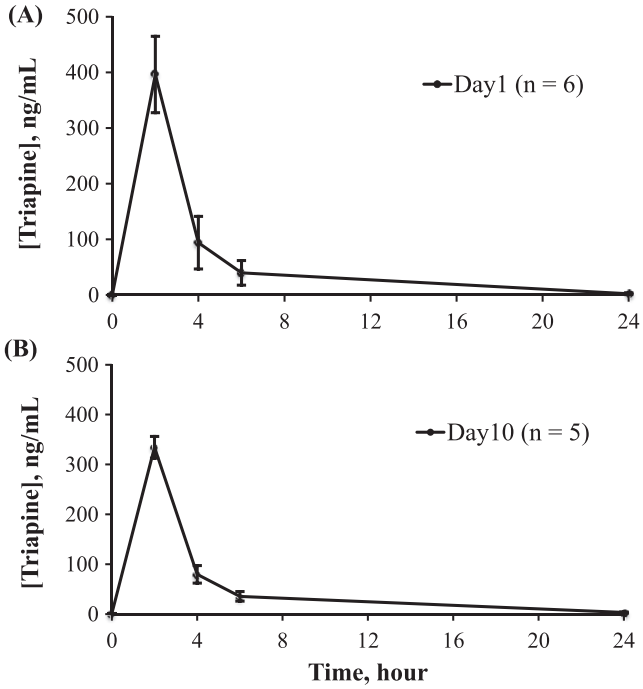


Figure 5. Mean triapine concentration time profiles of patients on days 1 and 10 by 2 h i.v. infusion of triapine at the dose of 25 mg/m^2 three times per week for 5 weeks.

Stability

The stability study was conducted for triapine and the IS, and the results were summarized in Table 6. At room temperature on the bench-top, tiapine and the IS stock solutions were stable for up to 24 h, whereas triapine and the IS in plasma were stable for at least 6 h. Triapine and the IS from plasma samples were stable in autosampler set at 4°C after sample preparation for 24 h. The recoveries of triapine and the IS after three freeze–thaw cycles were 96% for the low QC and 98% for the high QC, respectively, whereas the recovery of the IS was 92%. The studies also showed that there was no significant loss of triapine and the IS during long-term (30 days) storage at −20°C.

Application of the method

The feasibility of the LC-MS/MS method was demonstrated by the measurement of triapine concentrations in patient plasma samples collected from a previous phase I clinical trial of pelvic radiation, weekly cisplatin and triapine for locally advanced cervical cancer (Kunos *et al*., 2010a, 2010b). Figure 5 showed the triapine concentration–time profile in patients' blood samples on days 1 and 10 by 2 h triapine infusion at a dose of 25 mg/m^2 three times a week for 5 weeks. Compared with the HPLC-UV method used in the previous work (Kunos *et al*., 2010a, 2010b), the LC-MS/MS method developed not only could produce a comparable triapine concentration–time profile, but also was capable of measuring triapine concentrations (i.e. 0 h

on day 10, and 24 h on days 1 and 10) otherwise below the LLOQ of the HPLC-UV method (20.0 ng/mL). Therefore, the LC-MS/MS method is better suited for the clinical study of triapine, and has an unparalleled selectivity and LLOQ compared with the HPLC-UV method.

Conclusion

This work has provided a detailed discussion on the development and validation of an LC-MS/MS method for the quantitation of triapine in human plasma. The method employs a simple deproteinization protocol for sample preparation, a reversed-phase chromatograph for analyte separation and a tandem mass spectrometer for analyte quantitation. EDTA is used as anticoagulant and metal-ion chelator to prevent the unwanted complexation reaction between triapine and metal-ion interferents. This method has a linear calibration range of 0.250–50.0 ng/mL with the accuracy and precision well suited for analysis of human plasma samples, filling the gap between the needs of clinical trials and the less sensitive HPLC-UV method. It is useful for clinical studies of triapine in human.

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