Published in final edited form as:

Chin J Nat Med. 2015 July ; 13(7): 554–560. doi:10.1016/S1875-5364(15)30051-0.

Development and validation of an HPLC-MS/MS analytical method for quantitative analysis of TCBA-TPQ, a novel anticancer makaluvamine analog, and application in a pharmacokinetic study in rats

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Abstract

We have recently designed and synthesized several novel iminoquinone anticancer agents that have entered preclinical development for the treatment of human cancers. Herein we developed and validated a quantitative HPLC-MS/MS analytical method for one of the lead novel anticancer makaluvamine analog, TCBA-TPQ, and conducted a pharmacokinetic study in laboratory rats. Our results indicated that the HPLC-MS/MS method was precise, accurate, and specific. Using this method, we carried out *in vitro* and *in vivo* evaluations of the pharmacological properties of TCBA-TPQ and plasma pharmacokinetics in rats. Our results provide a basis for future preclinical and clinical development of this promising anticancer marine analog.

Keywords

TCBA-TPQ; Makaluvamine analogs; LC-MS/MS; Rat plasma; Pharmacokinetics

Introduction

Cancer poses a major health threat worldwide^[1,2], and chemotherapy plays an important role in the treatment of various human cancers. Even though targeted therapeutic drugs have provided better outcomes for certain cancer patients, natural products and their derivatives are still irreplaceable for cancer therapy and prevention^[3–5]. Due to their structural

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Conflict of Interest: These authors declare no conflict of interest.

biodiversity and chemical variations, natural products present a rich source of compounds for the discovery and development of new therapeutic and preventive drugs^[3–5]. Makaluvamines, a class of marine iminoquinone alkaloids isolated from sponges, have demonstrated *in vitro* and *in vivo* cytotoxicity against various types of cancers^[5, 6].

We have previously studied a series of makaluvamine analogs containing substitutions at the 7-position of the pyrroloiminoquinone ring^[6–12]. Numerous compounds were found to be active, including, 7-(benzylamino)-1, 3, 4, 8-tetrahydropyrrolo[4, 3, 2-de] quinolin-8 (1H)-one (BA-TPQ, Figure 1A) and 7-(4-fluorobenzylamino)-1, 3, 4, 8-tetrahydropyrrolo [4, 3, 2-de] quinolin-8(1H)-one (FBA-TPQ, Figure 1B), which were demonstrated to have anti-cancer activities both *in vitro* and *in vivo*, and their safety, mechanisms of action and pharmacological properties have indicated that they have great potential for development as new anti-cancer drugs^[6–12]. BA-TPQ was found to be a potent inducer of apoptosis and cell cycle arrest, and might have potential therapeutic applications for prostate cancers with varying genetic backgrounds (including the p53 status), and was active against breast cancer via mechanisms that were hormone-, Her2- and p53-independent. The ZAK- MKK4-JNK-TGF β signaling cascade was also found to be activated by BA-TPQ^[12].

Of interest, a new makaluvamine analog with substitutions at the 7-position of the pyrroloiminoquinone ring, 7-(4-chlorobenzylamino)-1-tosyl-1, 3, 4, 8-tetrahydropyrrolo [4, 3, 2-de] quinolin-8(1H)-one (TCBA-TPQ, Figure 1C), has been synthesized^[13]. Our studies have indicated that TCBA-TPQ has high potency against human cancer. In order to investigate the pharmacokinetics of this marine iminoquinone alkaloid, we established a fast and reliable HPLC-MS/MS method for the quantitation of TCBA-TPQ following administration to rats. We herein report the development and method validation and pharmacokinetic parameters of TCBA-TPQ.

Materials and Methods

Chemicals and reagents

TCBA-TPQ and the internal standard (IS), BA-TPQ, were synthesized as previously reported^[6,13]; the purity of both compounds was determined to be greater than 99%. Acetonitrile and methanol (LC-MS grade) were obtained from Merck (Darmstadt, Germany). Formic acid was purchased from Sigma (St Louis, MO, USA). Distilled, deionized water was produced by a Milli-Q reagent water system (Millipore, Inc, Bedford, MA). Blank rat plasma was obtained from the Laboratory Animal Center of Beijing Friendship Hospital (Beijing, China). All other chemicals and solvents used for the sample preparation and analysis were of analytical grade.

Instrumentation and analytical conditions

Instruments—The LC-MS/MS consisted of an Agilent 1200 liquid chromatography system (Agilent Technologies, Palo Alto, CA) equipped with a binary solvent delivery system, an autosampler and a column oven, and was connected to a 6460 triple quadrupole mass spectrometer (Agilent Technologies) equipped with an ESI interface.

Liquid chromatography—The chromatographic separation and quantification of the test compound was achieved using a SB-C₁₈ (1.8μm, 2.1 ×50 mm) analytical column (Agilent Technologies, CA, USA) protected by an Agilent gasket. The column temperature was maintained at 35°C. The system was set to use gradient elution at a flow rate of 0.4 mL·min⁻¹, increasing the acetonitrile-water ratio from 10:90 at 0 min to 50:40 at 1.0 min, keeping this ratio until 1.2 min, and then decreasing it to 10:90 in 0.1 min, then maintaining this ratio until the total running time of 3.5 min. There was 0.01% formic acid (v/v) in both the acetonitrile and water. The eluent was discharged to waste for the first 1.0 min to reduce the contamination of the flow into the ion source.

Mass spectrometry—The ESI source was operated in the positive ion mode with nitrogen as the nebulizer and drying gas. The final optimized ion source conditions were set as follows: temperature, 350 °C; gas flow, 12L·min⁻¹; nebulizer, 35 psi; capillary voltage, 4000 V; fragmentor, 150 V for TCBA-TPQ and 170 V for IS; collision energy, 29 V for TCBA-TPQ and 25 V for IS; and a dwell time of 500 ms. TCBA-TPQ and IS were monitored by multiple reaction-monitoring (MRM) using transitions as follows: m/z 466.1→311.1 for TCBA-TPQ and m/z 278→91.1 for IS, with a dwell time of 0.5s per transition (Table 1). All data were acquired and analyzed using the Agilent B.06.00 series software.

Standard solutions, calibration and quality control samples—Stock solutions of TCBA-TPQ (1 mg·mL⁻¹) and IS were prepared in methanol and stored at -20 °C. A series of standard working solutions with TCBA-TPQ concentrations of 40, 200, 1 000, 4 000, 16 000, and 40 000 ng·mL⁻¹ were obtained by further dilution of the stock solution with methanol. The standard curve concentrations were 2, 10, 50, 200, 800, and 2 000 ng·mL⁻¹ for the control (drug-free) pooled rat plasma. Quality control samples of low (5 ng·mL⁻¹), medium (200 ng·mL⁻¹) and high (1 600 ng·mL⁻¹) concentrations were prepared in the same way to assess the intra-day and inter-day precision and accuracy, as well as the matrix interference, sample recovery and sample stability.

Sample processing—For the sample preparation, rat plasma samples containing TCBA-TPQ (50 μL) and IS (5 μL, 1 000 ng·mL⁻¹) were placed into an eppendorf tube, and acetonitrile (200 μL) was used to precipitate proteins. The mixture was vigorously vortexed for at least 2 min, placed at 4 °C for 20 min and centrifuged at 18 000 g for 20 min at 4 °C. The supernatant was transferred into an autosampler vial, and 2 μL was injected into the LC-MS/MS system.

Determination of linearity, lower limit of detection, and lower limit of quantitation—The specificity of the method was assessed by analyzing blank samples spiked with TCBA-TPQ to observe the possible endogenous interference of plasma with the analytes. The linearity of the relationship between the detector response and TCBA-TPQ concentrations was confirmed within the range of 2–2 000 ng·mL⁻¹. Calibration curves were determined by plotting the peak area ratios of the analytes to the internal standard (IS) against the concentrations by the 1/x² weighted least-square linear regression. The lower limit of detection (LLOD) was defined as the peak signal of the compound equal to three

times the average noise level. The lower limit of quantification (LLOQ), defined as the lowest concentration on the calibration curve at which both precision and accuracy were less than or equal to 20%, was evaluated by analyzing samples prepared in six replicates on three consecutive validation days.

Determination of accuracy and precision—The accuracy and precision of the method were assessed by evaluating QC samples at three concentration levels (5, 200 and 1 600 ng·mL⁻¹) on four validation days (n = 5, 3, 3, 3, respectively) for intra-day and inter-day accuracy, respectively. The accuracy of the assay was assessed by comparing the calculated mean concentrations to the actual concentrations of serial dilutions. Standard deviations (SD) and the ratios of the standard deviation to the mean (RSD) were calculated and used to evaluate the precision. The accuracy and precision were required to be within ± 15%.

Determination of extraction recovery and matrix inference—The percentage recoveries of TCBA-TPQ at the three QC levels (5, 200 and 1 600 ng·mL⁻¹) from rat samples were determined by comparing the mean peak areas of extracted samples with those of pure compound dissolved in corresponding blank matrix after sample preparation. The absolute magnitude of matrix suppression at three QC levels levels (5, 200 and 1 600 ng·mL⁻¹) was determined by comparing the peak areas of the post-extraction spiked samples with those from unextracted analytes in acetonitrile at the same concentration.

Analysis of compound stability in vitro—The stability of the analytes in rat plasma was evaluated by analyzing QC samples at different concentrations (low, medium and high) under various conditions. The different types of stability evaluated were: freeze and thaw stability, which was assessed after three freeze-thaw cycles from -80 °C to room temperature; post-preparative stability, where extracted samples left in an auto-sampler for 24h were repeatedly analyzed; short-term stability, where QC samples were thawed and kept at room temperature for 6 h prior to analysis; and long-term stability, where QC samples were kept at -80 °C for 4 weeks and thawed at room temperature prior to the analysis. All of the samples were assessed by comparing the measured concentration after incubated in the noted conditions with the nominal concentrations, and the results were expressed as a percentage of the initial concentration. Samples were considered to be stable if the bias was within ± 15% of the nominal values.

Pharmacokinetic studies

Animals—Sprague-Dawley rats (weighing 180–220 g) were purchased from Vital River Laboratories (Beijing, China) and maintained on commercial diet and water *ad libitum* on an alternating 12h light/dark cycle. All experimental procedures carried out in this study were approved by the Beijing Friendship Hospital Animal Ethics Committee and followed the Guiding Principles for the Care and Use of Laboratory Animals.

Dosing and sampling—After an overnight fast, rats were intravenously administered TCBA-TPQ (5 mg·kg⁻¹) in PEG400: ethanol: saline (57.1%: 14.3%: 28.6%), and blood samples were obtained before administration and at 5, 15, and 30 min, and 1, 2, 3, 4, 6 and 8 h after dosing. A 120 µL blood sample was collected from the left femoral artery into a

heparinized tube for each time point. All blood samples were centrifuged at 3 000 rpm for 10 min, and a plasma sample (50 μ L) was precisely collected and stored at -80°C until analysis. All samples were then processed as described above, and concentration-time curves were obtained for the pharmacokinetic analysis.

Pharmacokinetic modeling—The average plasma concentrations of the drug following intravenous administration to animals were used for the pharmacokinetic analyses. Pharmacokinetic parameters were calculated by a two-compartmental model using the DAS2.0 software program^[14]. The maximum concentration (C_{max} , $\text{ng}\cdot\text{mL}^{-1}$), elimination half-life ($t_{1/2\beta}$, h), area under the concentration-time curve (AUC , $\text{ng}\cdot\text{h}\cdot\text{mL}^{-1}$), distribution volume (V_c , $\text{L}\cdot\text{kg}^{-1}$), and clearance (CL , $\text{L}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$) were calculated as applicable.

Results and Discussion

Optimization of the HPLC-MS/MS conditions and extraction methods

Quadrupole full scans were carried out in both positive and negative ionization modes to optimize the ESI conditions for TCBA-TPQ and IS. A higher intensity was observed in the positive ion mode. The full-scan mass spectra showed a predominantly protonated molecular ion $[\text{M}+\text{H}]^{+}$ at m/z 466.1 for TCBA-TPQ and m/z 278.0 for IS. The fragment ion, m/z 311.1, was dominant, and was therefore used for the quantification of TCBA-TPQ. Similarly, the optimum MRM transition of IS was chosen at m/z 278.0 $\rightarrow$ 91.1 (Figure 2).

The composition of the mobile phase played an important role in achieving good chromatographic behaviors, including good sensitivity, peak symmetry and appropriate resolution. It was found that a better peak shape and lower background noise were obtained using acetonitrile-water than methanol-water. Both the acetonitrile and water contained 0.1% formic acid to improve the MS detection and to provide better responses in the positive ionization mode. To improve the resolution and avoid contamination of the ion source, gradient elution was applied and the flow rate was optimized. As shown in Figure 3, both TCBA-TPQ and IS were eluted rapidly and selectively, with a good shape, and with retention times of approximately 2.48 and 1.81 min, respectively. We were able to develop a robust LC-MS/MS method which could finish detection of the target analyte within 3.5 min.

Reliable and fast sample preparation can improve the efficiency of an analysis, reduce the interference of impurities, and also ensure the extraction efficiency. In our previous studies of makaluvamine analogs, protein precipitation/extraction using acetonitrile was often used to obtain better purification^[11, 14]. In protein precipitation methods, the organic phase is often evaporated under nitrogen and then reconstituted by mobile phase. However, there have been studies that have eliminated the evaporation and reconstitution procedures, indicating that direct injection of the supernatant can also be used, and may help to prevent loss of the analyte^[15, 16]. In our method, four volumes of acetonitrile were used for precipitation, and no precipitation during chromatography or reduction of the mass response was found in the matrix after high speed centrifugation at low temperature.

Method validation

In the present study, we followed FDA guidelines to validate the HPLC-MS/MS methods, using the procedure as reported recently^[17]. No endogenous interference was observed at the retention times of TCBA-TPQ or IS due to the high selectivity of the MRM mode. Representative chromatograms of blank rat plasma (Figure 3A), control rat plasma (drug-free) spiked with TCBA-TPQ (Figure 3B) and IS (Figure 3C), and a plasma samples obtained from a rat after the intravenous administration of TCBA-TPQ (Figure 3D) are shown. The calibration curves for TCBA-TPQ were linear over the concentration range of 2–2 000 ng·mL⁻¹, and the typical regression equation was $Y = 8.0866X + 0.0278$ ($R = 0.9962$). The LLOD and LLOQ for the determination of TCBA-TPQ in plasma were found to be 0.5 and 2 ng·mL⁻¹, respectively. The intra-day and inter-day precision and accuracy of TCBA-TPQ were presented in Table 2. The intra-day and inter-day precision were both good, as expressed by the RSD of the observed concentrations of the QC samples, which were lower than 13.4% and 8.8%, respectively. The intra-day and inter-day accuracy, expressed as the mean of the observed concentrations of the QC samples, ranged from 100.4 to 111.8%. These results suggested that our method was accurate and reproducible for the determination of TCBA-TPQ in rat plasma.

The extraction recoveries were determined using five replicates of the QC samples at three concentration levels (5, 200, and 1 600 ng·mL⁻¹). As shown in Table 3, the mean extraction recoveries were in the range from 78.8–108.6% for TCBA-TPQ in plasma, indicating that the protein precipitation method offered good extraction efficiency for these analytes in the biological matrix. Five lots of blank plasma were tested for the absolute magnitude of matrix suppression by comparing standard made up in matrix extracted with a solvent standard. The bias for the matrix effects of TCBA-TPQ was found to be acceptable, showing that the extraction matrix had no endogenous substances that led to interference with the ionization of the analyte and IS.

Stability

At the designated time points, the samples were extracted and analyzed using the established analytical method. The data for the stability during sample processing are shown in Table 4, and indicated that TCBA-TPQ was stable through three repeated three freeze/thaw cycles, in an auto-sampler for 24 h, on the bench at room temperature for 6 h and after being frozen at –80 °C for 4 weeks. These results suggested that our methods for sample extraction, intermittent analysis and storage were valid and suitable for the sample analysis.

Pharmacokinetics in rats

To demonstrate the feasibility of the method, we assessed the concentration of TCBA-TPQ in the plasma of rats after the administration of a single intravenous dose (5 mg·kg⁻¹). Following the injection, the concentration of the compound in plasma was decreased rapidly, from $1\,244.37 \pm 121.33$ ng·mL⁻¹ at 5 min, to 438.47 ± 202.54 ng·mL⁻¹ at 1 h, 78.08 ± 60.80 ng·mL⁻¹ at 4 h, and to undetectable levels at 8 h (Figure 4). A two-compartment pharmacokinetic model was best described the concentration-time profiles of the intravenous administration of TCBA-TPQ. All parameters were summarized in Table 5.

These data provided a basis for future pharmacology studies of this compound as anticancer agent.

The present study represented our continued effort in developing novel anticancer cancer agents targeting oncogene^[18], including natural products^[6–13, 19–21] and synthetic compounds^[22–23]. The development and validation of sensitive, accurate, and precise analytical methods are the critical step in new drug discovery and development^[4, 24–27], especially for natural products^[4, 24–26]. Several of our marine analogs have entered the preclinical development for the treatment of human cancers^[6–14, 19–20]; the UPLC-MS/MS method developed for analysis of TCBA-TPQ and initial pharmacokinetic study would have a significant impact on future research and development of this agent and related analogs as well.

Conclusion

A rapid and sensitive HPLC-MS/MS method was developed and validated for determination of the plasma concentration of TCBA-TPQ. Furthermore, this method was applied to a pharmacokinetic study of TCBA-TPQ in intact rats, representing the first evaluation of the pharmacological characteristics of TCBA-TPQ *in vivo*. We believe that this robust method will facilitate further preclinical and clinical investigations of makaluvamine analogs.

Acknowledgments

Research Funding: This work was supported by the National Institutes of Health (NIH) grants R01 CA112029, R01 CA121211, and R01 CA186662 (to R.Z.) and 1UL1RR025777 (to S.V.). The contents are solely the responsibility of the authors, and do not necessarily represent the official views of the National Institutes of Health.

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Figure 1.
The structures of the makaluvamine analogs: (A) BA-TPQ; (B) FBA-TPQ; and (C) TCBA-TPQ.

Figure 2.
The product ion mass spectra of (A) TCBA-TPQ and (B) BA-TPQ (IS), and their respective structures

Figure 3. Representative multiple reaction monitoring chromatograms of (A) blank rat plasma, (B) plasma spiked with TCBA-TPQ (50 ng·mL⁻¹), (C) rats plasma spiked with TCBA-TPQ (50 ng·mL⁻¹) and IS (1 000 ng·mL⁻¹), (D) rat plasma obtained 15 min after the intravenous administration of 5 mg·kg⁻¹ TCBA-TPQ.

Figure 4.
The mean plasma concentration-time curve of TCBA-TPQ after the intravenous administration of 5 mg·kg⁻¹ to rats (n=5, mean ± SD).

Table 1

The optimized mass spectrometric conditions

Compound	Precursor ion (m/z)	Product ion (m/z)	Fragmentor (V)	Collision energy (V)	Polarity
TCBA-TPQ	466.1	311.1	150	29	Positive
BA-TPQ (IS)	278	91.1	170	25	Positive

Table 2

The precision and accuracy of the method for detection of TCBA-TPQ in rat plasma samples

QC (ng/mL)	Intra-day (n=5)		Inter-day (n=4)	
	Precision (% RSD)	Accuracy (mean, %)	Precision (% RSD)	Accuracy (mean, %)
5	6.1	100.4	8.1	111.8
200	6.6	104.6	8.8	105.9
1600	13.4	105.0	8.6	101.1

Table 3

Extraction recoveries and matrix factor effects for the detection method of TCBA-TPQ in rat plasma

QC (ng/mL)	Extraction recovery (n=5) (mean $\pm$ SD, %)	Matrix effect (n=5) (mean $\pm$ SD, %)
5	108.6 $\pm$ 15.4	114.4 $\pm$ 6.8
200	78.8 $\pm$ 4.2	106.5 $\pm$ 5.6
1600	83.4 $\pm$ 2.6	106.3 $\pm$ 2.3

Table 4

The stability of TCBA-TPQ in rat plasma

QC (ng/mL)	Percentage of TCBA-TPQ remaining (n=5) (mean $\pm$ SD, %)			
	Freeze-thaw cycles	Auto-sampler (24 h)	Room temperature	Long-term (–80°C, 4W)
5	105.4 $\pm$ 3.2	102.6 $\pm$ 6.0	100.9 $\pm$ 4.3	103.6 $\pm$ 7.2
200	108.2 $\pm$ 4.3	111.3 $\pm$ 4.5	110.6 $\pm$ 3.5	111.8 $\pm$ 6.5
1600	100.7 $\pm$ 2.3	109.8 $\pm$ 8.7	101.2 $\pm$ 3.8	104.9 $\pm$ 2.9

Table 5

The pharmacokinetic parameters of TCBA-TPQ in intact rats after the intravenous administration of a single dose of 5 mg/kg (n=5)

Parameters	Rats					mean ± SD
	No. 1	No. 2	No. 3	No. 4	No. 5	
C _{max} (ng/mL)	1181.24	1266.23	1343.56	1068.93	1361.87	1244.37 ± 121.33
T _{1/2} (h)	0.82	1.79	1.61	1.12	1.33	1.33 ± 0.39
V _c (L/kg)	0.007	0.003	0.002	0.005	0.003	0.004 ± 0.002
CL (L/h/kg)	0.002	0.003	0.002	0.002	0.003	0.002 ± 0.0005
AUC _(0-t) (ng/mL·h)	696.05	1582.12	2057.3	1871.46	1767.64	1594.91 ± 531.09
AUC _(0-∞) (ng/mL·h)	895.63	1941.02	2369.66	2102.78	1997.61	1861.34 ± 564.40

T_{1/2}: half-life of the compound; V_c: the volume of the central compartment; CL: clearance; AUC: area under the concentration-time curve