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Screening stabilisers for cyanoenone triterpenoid TX101 in rat plasma samples by simultaneous analysis of parent drug and the epoxidation product

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Abstract

In the development of bioanalytical methods, stabilizing drug molecules in biological matrices is crucial for ensuring reliable exposure data in pharmacokinetic and toxicokinetic sample analyses. This study focuses on the evaluation of stabilizing effects on the synthetic triterpenoid TX101, a cyanoenone triterpenoid Nrf2 activator with known instability in plasma samples. The molecule's unsaturated double bond is susceptible to oxidation, either nonenzymatically via oxygen or enzymatically through cytochrome P450 enzyme-catalyzed epoxidation. The research explores the impact of antioxidants (L-ascorbic acid, sodium metabisulfite, sodium sulfite) and P450 enzyme inhibitors (sodium diethyldithiocarbamate, memantine hydrochloride, 1-aminobenzotriazole) on TX101 stability in rat plasma samples. Results reveal that adding 2.5 mg/mL sodium sulfite or sodium metabisulfite effectively inhibits the nonenzymatic oxidation of TX101 to TX101-epoxide, while L-ascorbic acid shows minimal stabilizing effect. Among P450 enzyme inhibitors, sodium diethyldithiocarbamate and memantine hydrochloride exhibit modest stabilizing effects, likely attributed to their antioxidant activity. The developed High-formance liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) method, incorporating Supported Liquid Extraction for sample cleanup, allows simultaneous monitoring of TX101 and TX101-epoxide. Application of this method in a rat dose-range finding study confirms successful inhibition of TX101-epoxide formation in samples treated with sodium sulfite or sodium metabisulfite. Overall, the study emphasizes the importance of stabilizers in preventing nonenzymatic oxidation reactions during sample storage, providing valuable insights for bioanalytical method development and validation.

1 | INTRODUCTION

Cyanoenone triterpenoids are potent nuclear factor erythroid 2-related factor 2 (Nrf2) activators that have been identified for the potential treatment of oxidative and inflammatory stress-related diseases, including chronic kidney disease, liver disease, pulmonary hypertension, pulmonary arterial hypertension, and many other unmet therapeutic diseases such as neurovegetative disorders and metabolic diseases.[1] Synthetic oleanane triterpenoid Omaveloxolone (SKYCLARYS) as Nrf2 activator was recently approved by the FDA as the first drug for the treatment of Friedreich ataxia in adults and children 16 years of age and older.[2] As potent antioxidants,

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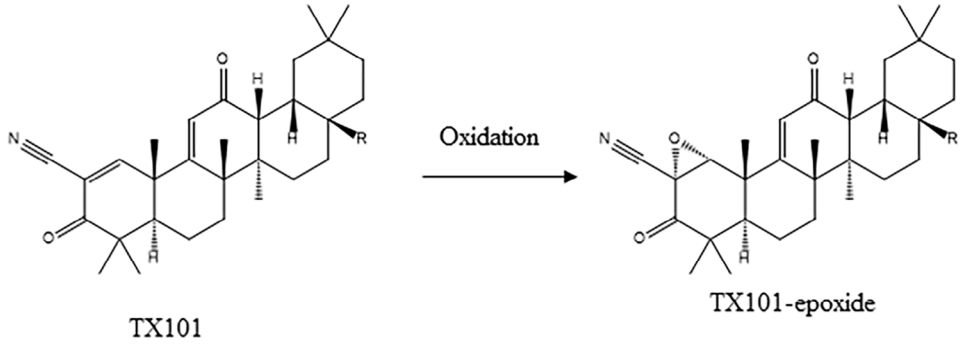

FIGURE 1 Oxidation of TX101 to TX101-epoxide.

cyanoenone triterpenoids are not stable in plasma samples and readily oxidised to epoxide form. Figure 1 shows the oxidation of the synthetic cyanoenone triterpenoid TX101 to TX101-epoxide. As a result, ensuring the stability of cyanoenone triterpenoids in biological matrices throughout the entire process of blood collection and processing, plasma storage, shipment, and analysis is of paramount importance. This is crucial for the development of robust bioanalytical methods that can support pharmacokinetic and toxicokinetic studies and provide reliable exposure data.

The instability of drugs in biological matrices is often affected by pH, temperature, light, oxygen, enzymes and occasionally anticoagulants.[3–5] Controlling the pH, temperature, and avoiding exposure to light are relatively easy to achieve. Adjusting blood pH to acidic or basic levels during sample collection is less favourable due to the potential for coagulation and hemolysis of erythrocytes. Consequently, pH adjustment is typically applied to plasma samples. However, this practice may not effectively prevent the degradation of drugs in whole blood during sample collection and centrifugation. The widely adopted approach to control temperature and prevent drug molecule degradation involves collecting blood on ice, promptly centrifuging the blood at 4°C, storing the resulting plasma samples at the lowest feasible temperature (e.g., −80°C), and maintaining the samples on dry ice during shipment to the bioanalytical lab for analysis. Photosensitive drug molecules necessitate the processing and storage of plasma samples under dark conditions. However, the previously mentioned approaches generally only slow down the reaction and may not completely stabilise the unstable drug molecules. Therefore, the addition of stabilisers, such as antioxidants or enzyme inhibitors in collection tubes, is often required to effectively protect unstable drug molecules.

The oxidation of cyanoenone triterpenoids can happen nonenzymatically (i.e., oxygen-induced auto-oxidation) or enzymatically such as cytochromes P450 catalysed olefin epoxidation, especially 2B4 and 2E1 isoforms.[6] This study aims to evaluate the stabilising effect of several commonly used antioxidants (L-ascorbic acid, sodium metabisulphite, and sodium sulphite) and known P450 enzyme inhibitors (sodium diethyldithiocarbamate, memantine hydrochloride and 1-aminobenzotriazole) on the stability of a cyanoenone triterpenoid compound TX101 in rat plasma samples.

2 | EXPERIMENTAL

2.1 | Chemicals, materials, and reagents

TX101, TX101-epoxide and d_3-TX101 were made at Reata Pharmaceuticals (Irving, TX, USA). ACS reagent sodium sulphite (≥ 98.0%), ReagentPlus sodium metabisulphite (≥ 99.0%), ACS grade L-ascorbic acid (≥ 99.0%), ACS reagent sodium diethyldithiocarbamate trihydrate, 1-aminobenzotriazole (≥ 98.0%), memantine hydrochloride (≥ 98.0%) were obtained from Sigma–Aldrich (St. Louis, MO). LC–MS grade acetonitrile and methanol were obtained from EMD Millipore (Burlington, MA). Optima LC/MS grade formic acid was obtained from Fisher Chemical (Fair Lawn, NJ). LC–MS reagent grade ethyl acetate was obtained from J.T. Baker (Rador, PA). Pooled K_3EDTA rat plasma was purchased from BioIVT (Hicksville, NY). Deionised water was made in house using a Barnstead GenPure xCAD Plus Ultrapure Water Purification System (Themo Scientific). Novum Supported Liquid Extraction (SLE) 96-well plate and 96-well collection plate (2-mL conical polypropylene) were purchased from Phenomenex (Torrance, CA). WatersAcqunity UPLC 700 mL round 96-well sample plate and silicone 96-well plate mat were obtained from Waters Corporation (Milford, MA). 96-Deepwell plate (1 mL) was obtained from Fisher Scientific (Pittsburg, PA).

2.2 | Sample preparation

2.2.1 | Preparation of spiking solution, quality control samples and calibration standards

Standard spiking solution and quality control (QC) spiking solution of TX101 and TX101-epoxide at a concentration of 40,000 ng/mL in methanol/water (1:1, v/v) were prepared by diluting 0.08 mL freshly prepared TX101 or TX101-epoxide stock solution (0.5 mg/mL in methanol) with 0.92 mL of methanol/water (1:1, v/v) and mixing well. Internal standard (IS) spiking solution at 50 ng/mL was also prepared in methanol/water (1:1, v/v) by diluting d_3-TX101 stock solution (0.5 mg/mL in methanol).

Sodium sulphite and sodium metabisulphite were prepared at a concentration of 200 mg/mL each by completely dissolving required

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amount of each chemical in deionised water and mixing well by vortexing. Sodium diethyldithiocarbamate trihydrate, 1-aminobenzotriazole and memantine hydrochloride were prepared at a concentration of 20 mg/mL by dissolving the required amount of each chemical in deionised water. The solutions were then sonicated for 10 min until it became clear. Plasma containing 50 mg/mL L-ascorbic acid was prepared by adding 0.5 g L-ascorbic acid into 10 mL K_3EDTA rat plasma and mixing well by vortexing until all L-ascorbic acid is dissolved. Plasma containing approximately 2.5 mg/mL of sodium sulphite or sodium metabisulphite was prepared by mixing 0.125 mL respective 200 mg/mL aqueous solution with 9.875 mL blank K_3EDTA rat plasma and mixing well by vortexing. Rat plasma containing approximately 2.5 mg/mL sodium diethyldithiocarbamate trihydrate or 1-aminobenzotriazole or memantine hydrochloride was prepared by mixing 1.25 mL respective 20 mg/mL aqueous solution with 8.75 mL blank K_3EDTA rat plasma and mixing well.

Three levels of QC samples, HQC (800 ng/mL), Middle QC (MQC) (40 ng/mL) and LQC (3 ng/mL) were prepared by serial dilution of the TX101 QC spiking solution (40,000 ng/mL) in rat plasma without stabiliser, containing 50 mg/mL L-ascorbic acid or 2.5 mg/mL of sodium sulphite, sodium metabisulphite, sodium diethyldithiocarbamate, 1-aminobenzotriazole and memantine hydrochloride, respectively. Only the LQC and HQC samples were used for stability evaluation. After the initial evaluation, eight levels of plasma standards of TX101 at 1, 2, 4, 15, 50, 250, 850 and 1,000 ng/mL were prepared by serial dilution of the TX101 standard spiking solution (40,000 ng/mL) in rat plasma containing 2.5 mg/mL sodium sulphite. A separate set of TX101-epoxide plasma standards (1–1,000 ng/mL) were also prepared and included in each analytical run to monitor the formation of TX101-epoxide of the stability samples in matrices with or without stabilising agent. An example of the preparation of QC samples and plasma standards is illustrated in Table 1.

2.2.2 | Sample extraction

TX101 and TX101-epoxide were extracted from rat plasma samples using a Novum 96-well SLE plate (part number 8E-S138-FGA). In brief, an aliquot of 25 µL rat plasma samples were pipetted into the well of a 1-mL 96-deepwell plate and 20 µL of 50 ng/mL d_3-TX101 in methanol/water (1:1, v/v) was added to each well using a repeat pipetter, except the blank sample where 20 µL of methanol/water (1:1, v/v) were added. After briefly mixing by vortexing, 300 µL of 0.2% formic acid in water was added to each well and mixed well by vortexing. The entire contents from each sample well, approximately 345 µL, were transferred into the corresponding well of a Novum 96-well SLE (solid phase extraction) plate using a multichannel pipettor. This SLE plate was positioned on top of a 2-mL conical polypropylene 96-well plate within a vacuum manifold setup. The sample solution was allowed to be completely adsorbed into the sorbent bed for 5 min after the initiation of the process by briefly applying vacuum, followed by the addition of ethyl acetate (0.6 mL × 2) to each well. The eluent was collected in the sample collection plate using gravity without vacuum which takes approximately 20 min. The sample collection plate was placed under a stream of nitrogen gas (40°C), until the sample was completely dried. The dried sample was reconstituted with 150 µL of acetonitrile/water/formic acid solution (40:60:0.1, v/v/v) and mixed well by vortexing for approximately 1 min. Subsequently, the samples were transfer to a new WatersAcqunity UPLC 700 mL round 96-well sample plate and capped with a 96-well silicone mat. The plate was placed in the High-performance liquid chromatography (HPLC) autosampler and an aliquot of the reconstituted sample was injected onto an LC–MS/MS system for quantitative analysis of TX101 and TX101-epoxide using d_3-TX101 as the internal standard.

2.3 | UPLC–MS/MS conditions

The UPLC–MS/MS system consisted of a Waters Acquity UPLC system and Xevo TQ-S triple quadruple mass spectrometer (Milford, MA). Chromatographic separation was conducted on an Acquity HSS C18 column (2.1 × 100 mm, 1.8 µm) coupled with an Acquity Vanguard HSS C18 guard column (2.1 × 5 mm, 1.7 µm). The column temperature was set at 40°C and autosampler temperature was set at 4°C. Mobile phase A was water/formic acid (100:0.1, v/v), and mobile phase B was acetonitrile/formic acid (100:0.1, v/v). The total flow rate was set at 0.4 mL/min. The gradient started from 40% B and increased to 80% B in 1 min, the reached 95% B within 3 min. After maintaining 95% B for 1 min, the gradient returned to the initial condition of 40% B at 4 min and equilibrated for 1 min before next sample was injected.

The mass spectrometer was operated in the electrospray position ion mode using the following optimised operating parameters: the ion spray voltage was 3.5 kV, desolvation temperature was 650°C, the desolvation gas flow was 1000 L/h, and the corn voltage and collision voltage was 54 and 40 V, respectively. Multiple reaction monitoring (MRM) transitions monitored were: *m/z* 530.4 → 163.1 (TX101), *m/z* 546.5 → 163.4 (TX101-epoxide) and *m/z* 533.4 → 163.2 (d_3-TX101).

2.4 | Plasma samples from a dose range-finding study of TX101 in Sprague–Dawley rat

A single-dose range-finding study of two formulations of TX101 (sesame oil vs. aqueous formulation containing 10% polysorbate 80 in deionised water) was conducted using Sprague–Dawley rats. Total 18 male rats were assigned to three groups (6 rats/group) in the study. Each animal in groups 1–3 received single PO gavage dose of 10 mg/kg (Group 1: sesame oil formulation), 10 mg/kg (Group 2: aqueous formulation) or 30 mg/kg (Group 3: aqueous formulation). Blood samples ($n = 3$ at each time point) were collected via sublingual vein at 1-, 2-, 4-, 8-, 12-, 24- and 48-h post-dose from 3 animals/group at alternating time points. Approximately, 0.5 mL blood was collected in K_3EDTA tube containing 0.0125 mL 100 mg/mL sodium sulphite (final concentration at 2.5 mg/mL). The blood samples were centrifuged at 2000 rpm for 10 min at 4°C, and the processed plasma samples were stored at −80°C until shipping to the bioanalytical laboratory for analysis.

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TABLE 1 Preparation of TX101 calibration standards and quality control samples in rat plasma by series dilution.

			Spiking scheme	
	Concentration (ng/mL)	Total volume (mL)	Rat plasma (mL)	ID and spiking volume (mL)
[a]STD. 8	1000	2.00	1.95	[c]Standard spiking solution, 0.050
STD. 7	850	1.00	0.15	STD.8, 0.850
STD. 6	250	1.00	0.75	STD.8, 0.250
STD. 5	50.0	2.00	1.90	STD.8, 0.100
STD. 4	15.0	1.00	0.70	STD.5, 0.300
STD. 3	4.00	1.00	0.92	STD.5, 0.080
STD. 2	2.00	2.00	1.92	STD.5, 0.080
STD. 1	1.00	1.00	0.50	STD. 2, 0.500
[b] HQC	800	2.00	1.96	[c]QC spiking solution, 0.040
MQC	40.0	1.00	0.95	HQC, 0.050
LQC	3.00	1.00	0.925	MQC, 0.075

Notes:
[a]Plasma standards were prepared in rat plasma containing 2.5 mg/mL sodium sulphite.
[b]Quality control samples (HQC, MQC and LQC) were prepared in rat plasma without stabiliser or containing respective antioxidants (50 mg/mL L-ascorbic acid, 2.5 mg/mL sodium sulphite, 2.5 mg/mL sodium metabisulphite) or cytochrome P450 inhibitors (2.5 mg/mL sodium diethyldithiocarbamate trihydrate, 2.5 mg/mL 1-aminobenzotriazole, 2.5 mg/mL memantine hydrochloride).
[c]The concentration of standard spiking solution and QC spiking solution concentration is 40,000 ng/mL in methanol/water (1:1, v/v).

3 | RESULTS AND DISCUSSION

The initial evaluation of TX101 stability in rat plasma samples involved comparing the peak area ratio (analyte/IS) of 3-h stability samples of low-quality control (LQC, 3.00 ng/mL) and high-quality control (HQC, 800 ng/mL) at room temperature and on ice to freshly prepared QC samples in the same matrix. These QC samples were prepared without stabilisers or with respective antioxidants or P450 enzyme inhibitors.

To minimise matrix interference and enhance throughput in an environment without automation, we adopted a supported liquid extraction (SLE) method using Novum 96-well SLE plates. The established LC–MS/MS methodology allows for the concurrent assessment of both TX101 and TX101-epoxide, enabling the tracking of TX101 degradation and the generation of TX101-epoxide in stability samples. Figure 2 shows representative chromatograms of a blank plasma sample spiked with internal standard (Figure 2A) and lower limit of quantification (LLOQ, 1.00 ng/mL) of TX101 (Figure 2B,) and TX101-epoxide (Figure 2C) in the matrix containing 2.5 mg/mL sodium sulphite. There is trace amount of non-deuterated impurity of TX101 in the internal standard d_3-TX101 and it is less than 20% of the LLOQ of TX101. The negligible interference at the retention time of TX101-epoxide is consistent and does not affect the quantification of TX101-epoxide.

After being kept at room temperature or on ice for 3 h, no decrease in TX101 was observed in QC samples prepared using rat plasma containing 2.5 mg/mL sodium sulphite or sodium metabisulphite. LC–MS/MS analysis also showed no presence of TX101-epoxide in those samples, indicating the stability of TX101 in plasma samples containing sodium sulphite or sodium metabisulphite. In contrast, a significant decrease in TX101 was observed in plasma samples containing no stabiliser and other antioxidants or P450 enzyme inhibitors (data not shown). As a result, freshly prepared rat plasma standards containing 2.5 mg/mL sodium sulphite were utilised to quantitatively assess the stabilising effect of other antioxidants and P450 inhibitors in QC samples. L-ascorbic acid, one of the most frequently used antioxidants in bioanalysis,[7–8] was initially evaluated at a concentration of 50 mg/mL in plasma samples. Table 2 summarises the stabilising effect of L-ascorbic acid (50 mg/mL) on the stability of TX101 at both room temperature and on ice. In QC samples without any stabilisers, there was an approximate −26% reduction in TX101. Interestingly, temperature had minimal impact on the stability of TX101, with a −25% reduction observed on ice compared to a −26% reduction at room temperature. While L-ascorbic acid at 50 mg/mL exhibited a slight stabilising effect compared to plasma samples without stabiliser, it did not completely stabilise TX101. Up to a −20% reduction was observed when samples were stored for 3 h at either room temperature or on ice.

Since temperature (room temperature vs. on ice) had a minimal impact on the stability of TX101, we conducted experiments evaluating the stability of TX101 at room temperature in QC samples containing 2.5 mg/mL sodium diethyldithiocarbamate, 1-aminobenzotriazole, memantine hydrochloride, respectively, using freshly prepared standard curves in rat plasma containing 2.5 mg/mL sodium sulphite. For confirmation purpose, six replicates of LQC and HQC samples prepared in rat plasma with and without stabiliser (2.5 mg/mL sodium metabisulphite) were also included in the analytical run (Figure 3). As previously observed, TX101 is not stable in plasma samples without any stabiliser, approximately −25% reduction was observed after 3 h at room temperature (Figure 3A). Certain isozymes of P450 enzymes have been reported to be present in plasma exosomes.[9] In this study, we assessed the stabilising effect of 1-aminobenzotriazole

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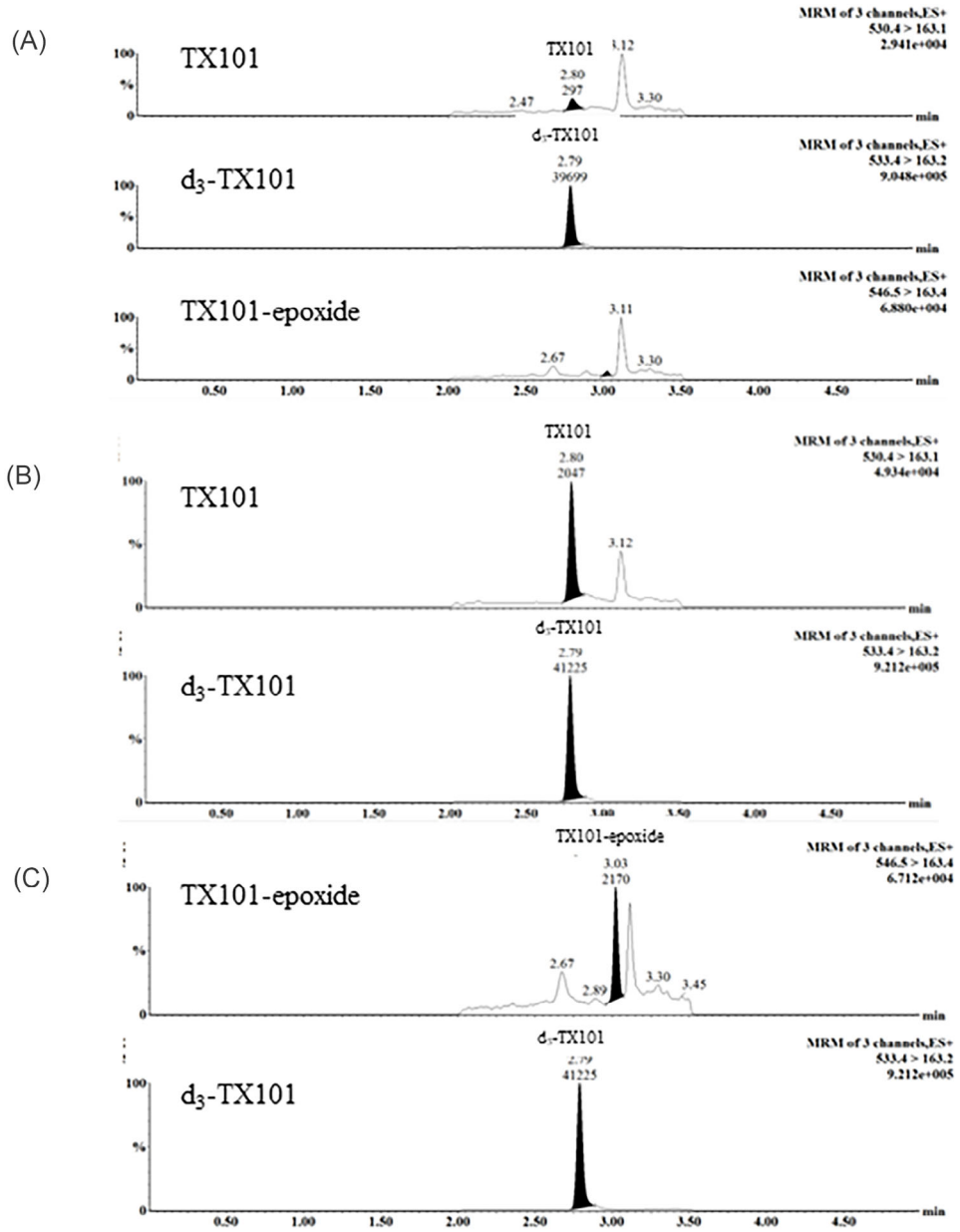

FIGURE 2 Representative LC–MS/MS chromatograms of (A) blank sample spiked with internal standard; (B) lower limit of quantification of TX101 at 1.00 ng/mL; and (C) lower limit of quantification of TX101-epoxide at 1.00 ng/mL.

(ABT), a known nonselective cytochrome P450 enzyme inhibitor.[10–12] The negligible stabilising effect of ABT on TX101 in the current study is interesting and suggests that the oxidation of TX101 is unlikely to be attributed to P450 enzymes (Figure 3B). Both memantine and sodium diethyldithiocarbamate have been reported to possess the antioxidant activity and the ability to selectively inhibit certain P450 isozyme enzymes.[13–16] The moderate stabilising effect of both chemicals on TX101, as illustrated in Figure 3C,D, further suggests that their impact is mediated by antioxidant activity rather than a P450 inhibitory effect. Plasma concentration of 2.5 mg/mL sodium metabisulphite successfully stabilised TX101, with the %bias of LQC and HQC samples within ± 6% of the nominal concentration after 3 h of storage at room temperature (Figure 3E). Furthermore, LC–MS/MS analysis did not detect the presence of TX101-epoxide in any of the sodium metabisulphite-containing LQC and HQC samples, confirming the stabilising effect of sodium metabisulphite.

The performance of the developed method was further demonstrated during analysis of plasma samples from a single-dose range-finding study comparing two formulations. All standards and QC samples met the acceptance criteria (%CV, 15%; %Bias within 15%; 20% for LLOQ) in the analytical run. Figure 4 shows the PK profile of TX101 in male Sprague–Dawley rats. The absence of TX101-epoxide in all the samples further confirms that the addition of sodium sulphite at a concentration of 2.5 mg/mL in the blood collection tube effectively stabilised TX101, and the ex vivo epoxidation of TX101 is most likely a result of nonenzymatic oxidation.

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TABLE 2 L-Ascorbic acid on the stability of TX101 in rat plasma samples (3 h).

	Nominal concentration (ng/mL)	Mean ($n = 6$) measured concentration (ng/mL)	%CV	%Bias
LQC	3.00[a]	2.22	15.9	−26.1
	3.00[b]	2.23	3.5	−25.7
	3.00[c]	2.40	4.4	−19.9
	3.00[d]	2.38	2.9	−20.5
HQC	800[a]	588.57	1.3	−26.4
	800[b]	601.68	2.3	−24.7
	800[c]	640.37	1.3	−19.9
	800[d]	661.68	2.8	−17.9

Notes:
[a]Rat plasma at room temperature.
[b]Rat plasma on ice bath.
[c]Rat plasma containing 50 mg/mL L-ascorbic acid at room temperature.
[d]Rat plasma containing 50 mg/mL L-ascorbic acid on ice bath.

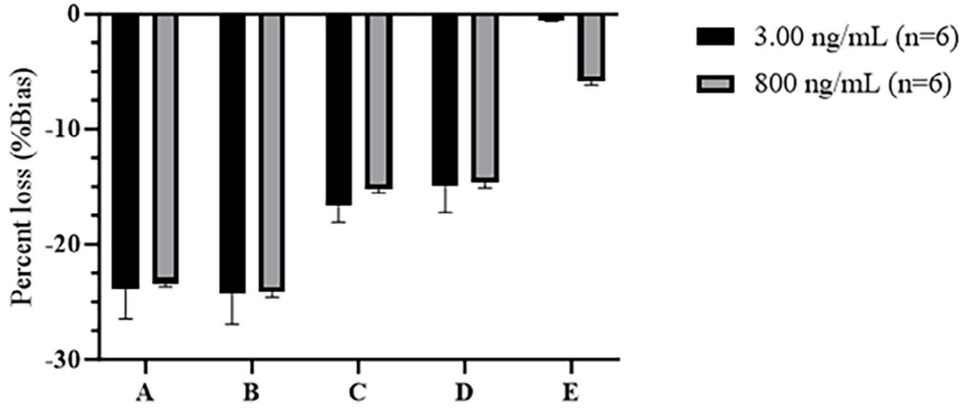

FIGURE 3 Effect of antioxidants and P450 enzyme inhibitors on the stability of TX101 in rat plasma after 3 h at room temperature. (A) No stabiliser; (B) plasma containing 0.25% 1-aminobenzotriazole; (C) plasma containing 0.25% sodium diethyldithiocarbamate; (D) plasma containing 0.25% memantine hydrochloride; and (E) plasma containing 0.25% sodium metabisulphite.

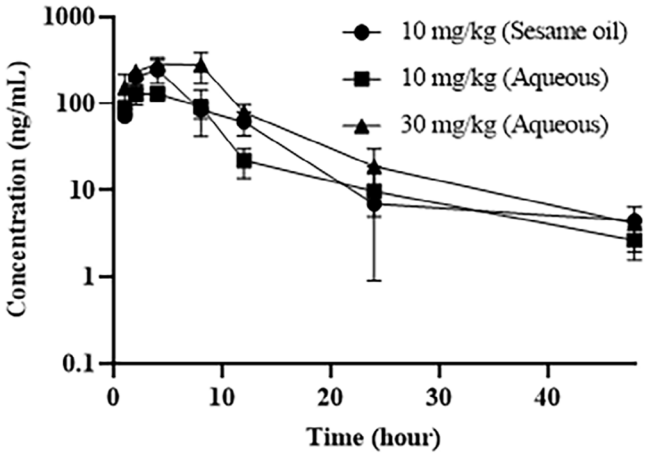

FIGURE 4 Mean TX101 concentrations in male Sprague–Dawley rats after single-dose administration of TX101 in sesame oil (10 mg/kg) and in aqueous formulation (10 and 30 mg/kg).

4 | CONCLUSION

We evaluated the impact of various antioxidants and P450 enzyme inhibitors on the stability of the synthetic cyanoenone triterpenoid TX101 in rat plasma samples. The LC–MS/MS method developed for simultaneous analysis of both TX101 and its epoxidation product allowed us to assess TX101 stability in biological matrices while concurrently monitoring potential epoxide formation.

Our findings demonstrate that TX101 in rat plasma samples can be effectively stabilised by using 2.5 mg/mL sodium sulphite or metabisulphite, whether the samples are stored at room temperature or on ice. Additionally, our data indicate that the epoxidation of TX101 in rat plasma is a nonenzymatic oxidation reaction occurring ex vivo during sample collection, transportation, or processing. As a result, it is recommended that in-life study sites collect blood samples in tubes containing sodium sulphite at a final concentration of 2.5 mg/mL.

Furthermore, the developed method was successfully transferred to a bioanalytical Contract Research Organization (CRO), where it

underwent full validation to support Good Laboratory Practice (GLP) toxicity and pharmacokinetic studies.

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CONFLICT OF INTEREST STATEMENT

Qingguo Tian and Edward Tamer are employed by and have a financial interest in Reata Pharmaceuticals, Inc. Lynn Tian is a Chemistry undergraduate student at Stony Brook University conducting a summer internship at Reata Pharmaceuticals.

DATA AVAILABILITY STATEMENT

Data are available from the corresponding author upon request.

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