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Development of a HPLC method for the quantification of Docetaxel in the BPMO@DTX injectable formulation

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ABSTRACT

Biodegradable BPMO@DTX (B@D) nanoparticles were successfully synthesized, exhibiting potent anticancer activity while mitigating the systemic toxicity of docetaxel (DTX). To advance the development of an injectable B@D nanoformulation, an accurate and robust method for DTX quantification was required. High-performance liquid chromatography (HPLC) was employed, with sample preparation based on a biodegradation technique, Glutathione (GSH-induced degradation), which was selected as the optimized procedure. Ultrasonication was evaluated only during the method development stage as a comparative approach. Key parameters—including solvent type, sample-to-solvent ratio, and biodegradation time—were optimized, and the method was validated according to International Conference on Harmonization Q2(R^2) guidelines. The optimized HPLC conditions comprised a mobile phase of acetonitrile:water (60:40, v/v), PDA detection at 230 nm, a Gemini 5 µm C18 (250 × 4.6 mm) column, 30°C temperature, 1.2 ml/minute flow rate, and 20 µl injection volume, all meeting validation requirements. Ultrasonication resulted in only ~30% DTX recovery, rendering it unsuitable for quantification. In contrast, the biodegradation method—incubating B@D with 20 mM GSH for 48 hours, followed by dichloromethane extraction (1:5 ratio, 5 minutes, 6,000 rpm, repeated five times)—achieved >99% recovery. This validated method provides a reliable analytical tool for DTX quantification in B@D formulations, supporting further pharmaceutical development.

1. INTRODUCTION

Docetaxel (DTX), a second-generation anticancer agent of the taxane family, acts by binding to β-tubulin, thereby inhibiting microtubule assembly and disrupting cell division [1–3]. It is approved for treating various cancers, including prostate, non-small cell lung, and breast cancers [4]. However, DTX has very low aqueous solubility (6–7 µg/ml at 25°C), leading to poor oral bioavailability and necessitating parenteral administration. Current injectable formulations require solubilizing agents such as ethanol and Tween 80, which can cause hypersensitivity reactions and neurotoxicity [5].

To address these issues, nanotechnology-based drug delivery systems have been widely investigated to improve solubility, enhance bioavailability, and reduce systemic toxicity [6–8]. Among these, mesoporous silica nanoparticles (MSNs) stand out for their ease of synthesis, high stability, and excellent biocompatibility [9,10]. However, concerns persist regarding their long-term toxicity, particularly their prolonged circulation and slow clearance. Notably, nanoparticles smaller than 5 nm are eliminated more readily [11]. Building on this, our team

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developed a novel synthesis approach to produce biodegradable MSNs incorporating tetrasulfide linkages [12].

Through optimized reaction conditions, a novel biodegradable MSN—termed biodegradable periodic mesoporous organosilica (BPMO)—was synthesized [6]. This biodegradable design is expected to reduce the potential long-term toxicity of silica-based nanocarriers. DTX was then loaded into BPMO nanoparticles, yielding B@D. *In vitro* studies showed that B@D significantly enhanced cytotoxicity against cancer cells while lowering toxicity to normal cells, suggesting its potential as a safer, more effective anticancer formulation [6].

To further develop an injectable B@D formulation, accurate quantification of DTX within the nanoparticles is essential. This is challenging due to the high structural stability of BPMO under physiological conditions—a trait shared with conventional MSNs—which limits drug release and complicates quantification [13]. Moreover, standard pharmacopeial methods, United States Pharmacopeia (USP, BP), are not directly applicable to nanoparticle formulations. Therefore, a tailored, sensitive, and specific analytical method is needed to accurately determine DTX content in B@D. To the best of our knowledge, no validated method has been reported for this purpose.

This study presents, to the best of our knowledge, the first fully validated high-performance liquid chromatography (HPLC) method for quantifying the total amount of DTX loaded into B@D injectable formulations. Unlike previous approaches focusing mainly on drug release profiles from biodegradable polymers or mesoporous silica, our method addresses the unique analytical challenges of the robust tetrasulfide-bridged BPMO framework, where conventional extractions achieve only ~30% recovery. We developed a two-step sample preparation involving accelerated Glutathione (GSH)-induced degradation (20 mM, 48 hours) followed by optimized multiple dichloromethane (DCM) extractions (1:5 v/v, 5 min × 5), achieving >99% recovery with excellent reproducibility. The method was fully validated according to International Conference on Harmonization (ICH) Q2(R^2), and provides high specificity in the presence of matrix degradation products, offering a robust tool for quality control of B@D injectable nanoformulations. In addition, unlike the USP monograph method for DTX, which is designed for conventional injections and does not account for mesoporous carriers, the present assay incorporates a redox-triggered degradation step, optimized extraction, and full ICH Q2(R^2) validation. These improvements extend beyond the compendial assay and ensure reliable quantification of DTX in B@D nanoformulations.

2. MATERIALS AND METHODS

2.1. Materials

The analytical grade reagents and materials used in this study were obtained from commercial suppliers and used without further purification: acetonitrile (ACN) for spectroscopy (LiChrosolv®, Merck, Germany), DCM for spectroscopy (Uvasol®, Merck, Germany), dimethyl sulfoxide (DMSO) for spectroscopy (Uvasol®, Merck, Germany), phosphate-buffered saline (PBS) pH 7.4 (Gibco™), docetaxel standard (Sigma–Aldrich), absolute ethanol (EMSURE®, Merck, Germany), GSH (Gracious Organic LLP), poloxamer F-127 (poloxamer 407) (BASF, Germany), polyethylene glycol (PEG) 400 (Dow Chemical), and Milli-Q water. The components of the nano-injectable formulation included B@D equivalent to 1 mg of DTX, poloxamer 127 (6 mg), PEG 400 (40 mg), and water to a final volume of 1 ml. The chromatographic analyses were performed on a Shimadzu LC-2030C 3D system equipped with a PDA detector, using a Gemini 5 µm C18 110 Å column (250 × 4.6 mm). The formulation was stored at temperatures below 30°C in a transparent type 1 glass vial with a rubber stopper. The B@D raw material sample was synthesized by the research team in a previous study, with an average DTX loading capacity of approximately 122.25 mg per gram of BPMO, as previously reported [6]. This value reflects the initial physicochemical characterization of the synthesized batch and was not directly applied in the present HPLC quantification, where the DTX content was re-determined using the validated analytical method. The sample was stored at temperatures below 30°C and a relative humidity of 75% ± 5%.

2.2. Preparation of standard sample, test samples, and placebo

Diluent: A mixture of acetonitrile and water (50:50, v/v) was used.

Standard solution: A standard solution of DTX at 0.2 mg/ml in diluent was prepared by accurately weighing 1 mg of DTX standard into a 5 ml volumetric flask. Two milliliters of diluent were added, and the mixture was sonicated for 10 minutes, allowed to cool, and then made up to volume with diluent before being mixed thoroughly. The solution was filtered through a 0.45 µm membrane filter. This sample preparation procedure was adapted from the current USP monograph, with modifications to suit the experimental conditions of the research group [14].

Test sample: The solution of nano-injection formulation (1 ml) was dispersed uniformly into 50 ml of PBS buffer (pH 7.4) containing 20 mM GSH. The dispersion was stirred continuously at 400 rpm for 192 hours at 37°C, with sonication applied for 30 minutes every 24 hours of stirring. DCM was added to the sample at a 5:1 ratio (1/5 volume of DCM per addition), followed by shaking at 700 rpm for 10 minutes and centrifugation at 6,000 rpm at 20°C to collect the DCM phase. This process was repeated five times. All DCM extracts were combined and allowed to evaporate naturally to dryness, and the resulting residue was collected. The residue was dissolved in 5 ml of diluent, sonicated for 5 minutes, and filtered through a 0.45 µm membrane filter.

Placebo: The placebo consisted of the nano-injection formulation containing only BPMO without DTX, prepared using the same procedure as the test sample.

2.3. Sample processing procedures

2.3.1. Sonication method

The principle of the sonication method is based on the study by Manzano and Vallet-Regí [15], which employed ultrasonic waves to facilitate the release of Ibuprofen from

MSM-41 nanoparticles during treatment. With the anticipation that BPMO nanoparticles exhibit ultrasound-responsive behavior during DTX release, various sonication experiments were conducted as follows: One milliliter of the nano-injection formulation was accurately pipetted into a porcelain dish and dried in a static oven at 60°C for 2 hours to obtain the residue. The residue was dispersed in 1 ml of DCM, sonicated for 20 minutes at 30°C, and then centrifuged at 10,000 rpm for 20 minutes at 20°C. The supernatant was collected, evaporated naturally, and the resulting white residue was obtained. This residue was dissolved in 5 ml of diluent, sonicated for 5 minutes, filtered through a 0.45 µm membrane filter, and subjected to quantification. The procedure was repeated for four additional experiments, with the sonication time increased by 20 minutes for each subsequent experiment. The results of the five experiments were compared based on the quantified DTX values obtained.

2.3.2. Redox method (biodegradation of BPMO)

Mai *et al.* [16] demonstrated that BPMO undergoes biodegradation in PBS buffer (pH 7.4) supplemented with 10 mM GSH. Based on Dat's study, to facilitate the degradation of BPMO for DTX extraction, the research group employed PBS buffer (pH 7.4) combined with GSH for investigation.

Solvent selectivity: To select an appropriate extraction solvent, solvent groups used for investigation include: DCM, DMSO, and ethanol absolute [17]. Experimental procedure: 1 ml of the nano-injection formulation was dispersed evenly into 5 ml of extraction solvent, sonicated for 25 minutes, shaken at 700 rpm for 10 minutes, and centrifuged at 6,000 rpm for 10 minutes at 20°C. The entire supernatant obtained after centrifugation was collected and transferred to a 50 ml Eppendorf tube. It was then allowed to evaporate naturally to obtain the residue. Five milliliters of diluent were added to the residue, sonicated for 5 minutes, filtered through a 0.45 µm membrane filter, and analyzed by HPLC. The results were compared to determine the solvent that provided the best extraction efficiency for DTX, which was subsequently selected for the next stage of investigation. Extraction efficiency was determined by comparing the amount of DTX recovered after extraction with the known initial DTX content of the B@D formulation. The recovery percentage was calculated as: (amount of DTX recovered/theoretical amount of DTX in the sample) × 100%. The solvent yielding recovery values within 98.0%–102.0% was considered optimal.

Solvent ratio and extraction time: After selecting the optimal solvent, we investigated the best sample-to-solvent ratio and extraction duration. Ratios (v/v) tested were 1:1, 1:3, 1:5, and 1:7. Extraction durations included: (i) a single 25-minute extraction using the full solvent volume, or (ii) five consecutive 5-minute extractions, each with one-fifth of the total solvent volume. Procedure: One milliliter of the nano-injection formulation was dispersed in 50 ml of PBS buffer (pH 7.4) containing 20 mM GSH and stirred at 400 rpm for 192 hours at 37°C, with 30-minute sonication applied every 24 hours. Solvent was added at the specified ratios, shaken at 700 rpm for the set duration, and centrifuged at 6,000 rpm at 20°C to collect the extract. For multiple-extraction samples, all extracts were combined. The combined solutions were evaporated to dryness, and the residue was dissolved in 5 ml of diluent, sonicated for 5 minutes, filtered through a 0.45 µm membrane, and quantified. The optimal ratio and extraction time were selected based on recovery rates within 98.0%–102.0% of the initial B@D content.

Degradation time according to GSH concentration: Compared to the 10 mM GSH concentration used by Mai *et al.* [16], in this study, to accelerate the degradation rate of B@D (and shorten the experimental time), higher concentrations of GSH were selected for investigation, specifically 10 mM, 20 mM, and 30 mM. Experimental procedure: The nano-injection formulation was dispersed evenly into 50 ml of PBS buffer (pH 7.4) containing GSH at the investigated concentrations. The suspension was stirred continuously at 400 rpm for 192 hours at 37°C (with the container covered to prevent evaporation), with sonication applied for 30 minutes every 24 hours of stirring. Ten milliliters of the sample were withdrawn at 24, 48, 72, and 168 hours, and the remaining suspension was collected at the final time point (192 hours). The extraction solvent was added to the samples at the tested ratios, shaken at 700 rpm for the specified extraction time, and centrifuged at 6,000 rpm at 20°C to collect the extract. The extract was allowed to evaporate naturally to obtain the residue. The residue was dissolved in 5 ml of diluent, sonicated for 5 minutes, and filtered through a 0.45 µm membrane filter. The resulting solutions were analyzed by HPLC. Based on the analytical results, the appropriate degradation time was evaluated as a function of GSH concentration and incubation duration.

Conclude the suitable parameters for the biodegradation process and extraction of the B@D nano-injection sample.

2.4. Optimization of chromatographic parameters

The chromatographic conditions were developed based on the current version of the USP, with modifications tailored to the specific requirements of this study [14]. The HPLC system used was the Shimadzu LC-2030C 3D, equipped with a PDA detector set at a wavelength of 230 nm. The chromatographic separation was achieved using a Gemini 5 µm C18 110 Å column (250 × 4.6 mm). This was the only column employed throughout method development, optimization, and validation, and no additional columns were evaluated. The

Table 1. The investigated mobile phase ratios.

Conditions	Percentage (%)	
	Acetonitrile	Water
Condition 1	40	60
Condition 2	45	55
Condition 3	55	45
Condition 4	60	40
Condition 5	65	35
Condition 6	70	30
Condition 7	75	25
Condition 8	80	20
Condition 9	85	15

column temperature was varied at 25°C, 30°C, and 35°C, while the flow rate was tested at 0.8, 1.0, 1.2, and 1.4 ml/minute. The injection volume was adjusted to 10 µl, 20 µl, and 30 µl. The mobile phase ratios were investigated according to the isocratic program, as detailed in Table 1. Based on the results obtained, the mobile phase ratio was selected to meet specific criteria: the peaks should be symmetrical without peak splitting, shoulders, or tailing, and should not exhibit any anomalies; the retention time should be optimal for time efficiency; the asymmetry factor (AS) was required to fall within the range of $0.8 \leq AS \leq 1.5$; and the number of theoretical plates (N) should be suitable for accurate and reliable quantification. Resolution (Rs) between the DTX peak and the nearest eluting component was also monitored as part of system suitability, and was consistently greater than the USP acceptance criterion of 2.0.

2.5. Validation of the analytical procedure

According to the ICH Q2 (R^2) guideline for the validation of analytical procedures, several parameters must be validated for quantitative analysis, including accuracy, precision, specificity, linearity (Table 2), and the determination range [18]. Specificity is evaluated by comparing chromatograms of the standard sample, test sample, placebo, and blank, ensuring that the DTX peak is sharp and symmetrical in the standard sample, absent in the placebo and blank, and matching the retention time of the standard in the test sample. System suitability is tested by injecting the standard solution six times into the HPLC system, ensuring stable operation with relative standard deviation (RSD) values for retention time, peak area, asymmetry factor, and theoretical plates all ≤ 2%. Linearity is determined by constructing a linear regression equation from standard DTX samples, requiring a correlation coefficient (R^2) of ≥ 0.998. For clarity, the concentration range used for linearity (0.12–0.32 mg/ml) refers to the injected working standard solutions, corresponding to 60%–160% of the nominal assay level. The nominal 100% assay concentration of the test sample was expressed as 1 mg/ml prior to chromatographic dilution, which falls within this validated linear range. Accuracy was evaluated by spiking known amounts of DTX reference standard into the placebo matrix (BPMO without drug) at three concentration levels (80%, 100%, and 120% of the nominal concentration). Recovery rates were calculated, with acceptable limits defined as 98%–102% and RSD ≤ 2%. Precision is evaluated in terms of repeatability and intermediate precision, with RSD values ≤ 2% for both. Finally, the determination range is defined based on the results of accuracy, linearity, and precision studies.

2.6. Statistical analysis

Statistical calculations and analyses were conducted using Microsoft Office Excel 2016 (Version 2102, Build 13801.20266, 64-bit). The results are expressed as the mean ± standard deviation (SD). T-tests were employed to compare two mean values, with statistical significance set at a p-value of < 0.05. All experiments were performed in triplicate, and the average values were calculated.

Data are presented as mean ± SD (n = 3 unless otherwise specified). One-way ANOVA was applied for comparisons involving three or more groups (solvent type, sample:solvent ratio, GSH concentration at 48 hours). Pairwise comparisons were conducted using Welch's t-tests with Bonferroni adjustment. Welch's t-test was used for two-group comparisons (extraction schedules). A two-sided $p < 0.05$ was considered statistically significant. F-values, degrees of freedom, p-values, and 95% confidence intervals are reported in the Results section.

Table 2. Docetaxel concentration range used for linearity validation (60%–140%, corresponding to 0.12–0.28 mg/ml).

Samples	DTX percentage (%)	DTX concentration (mg/ml)
1	60	0.12
2	80	0.16
3	100	0.2
4	120	0.24
5	140	0.28

3. RESULTS AND DISCUSSION

3.1. Optimization of chromatographic parameters

The results of the investigation on the composition and ratio of the mobile phase are presented in Table 3. Based on the experimental conditions, the Gemini 5 µm C18 110 Å LC column (250 × 4.6 mm) was selected for the study. As shown in Table 3, increasing the proportion of ACN relative to water resulted in a gradual decrease in the retention time of the peak. The variation in ACN:water ratio directly affected the retention time of DTX. As the proportion of ACN increased, retention time decreased, which aligns with the low polarity of DTX and its greater solubility in organic solvents [19]. However, an increased ACN proportion also led to a gradual reduction in the number of theoretical plates, suggesting compromised column efficiency. Since theoretical plate count reflects chromatographic resolution, ratios above 60:40 failed to meet the predefined criterion of 7.000–10.000 plates [20]. Therefore, an ACN:water ratio of 60:40 was selected as optimal, balancing adequate elution with acceptable column performance.

Regarding the flow rate, increasing it from 0.8 ml/minute to 1.4 ml/minute resulted in a gradual decrease in retention time, with a corresponding reduction in the number of theoretical plates. At a flow rate of 1.2 ml/minute, all parameters met the required criteria. Although most tested values yielded consistent results, a decline in theoretical plate number was observed at 1.4 ml/minute. To optimize runtime without sacrificing separation efficiency, a flow rate of 1.2 ml/minute was selected.

Regarding column temperature, no significant differences were observed between the temperatures studied, ranging from 25°C to 35°C. Therefore, to save time, a temperature of 30°C was chosen, which significantly reduced the equilibration time required for the HPLC system to reach the desired temperature.

For injection volume, increasing the sample injection volume resulted in a decrease in the asymmetry factor, with a risk of column overload. At both low (10 µl) and high (30 µl)

Table 3. Results of the mobile phase composition and ratio investigation.

Experiments		Retention time (minutes)	Peak area	Asymmetry factor (A_S)	Number of theoretical plates
Mobile phase rates (ACN:water v/v)	40 : 60	28.481 ± 1.204	3,925,728 ± 161,653	1.075 ± 0.009	10,179 ± 430
	45 : 55	15.427 ± 0.489	3,906,288 ± 131,692	1.063 ± 0.006	9,578 ± 304
	55 : 45	6.921 ± 0.338	3,919,174 ± 190,170	1.072 ± 0.010	9,125 ± 387
	60 : 40	5.406 ± 0.159	3,949,301 ± 114,946	1.093 ± 0.005	8,885 ± 232
	65 : 35	4.481 ± 0.185	3,944,661 ± 176,809	1.115 ± 0.008	6,634 ± 344
	70 : 30	3.880 ± 0.184	3,936,454 ± 127,796	1.136 ± 0.007	6,457 ± 238
	75 : 25	3.463 ± 0.114	3,946,337 ± 186,672	1.160 ± 0.011	5,532 ± 154
	80 : 20	3.220 ± 0.145	3,945,125 ± 105,757	1.173 ± 0.006	5,249 ± 216
Flow rates (ml/minute)	0,8	6.683 ± 0.088	5,660,987 ± 267,351	1.095 ± 0.008	9,385 ± 341
	1.0	5.388 ± 0.060	4,527,720 ± 162,145	1.102 ± 0.007	9,157 ± 387
	1.2	4.498 ± 0.043	3,781,065 ± 155,476	1.100 ± 0.010	8,786 ± 256
	1.4	3.876 ± 0.055	3,245,579 ± 95,412	1.602 ± 0.009	5,985 ± 355
Column temperature(°C)	25	4.542 ± 0.196	3,789,750 ± 152,649	1.103 ± 0.008	8,685 ± 361
	30	4.498 ± 0.165	3,781,065 ± 140,973	1.100 ± 0.010	8,875 ± 331
	35	4.450 ± 0.125	3,777,278 ± 101,243	1.098 ± 0.010	8,756 ± 388
Injection volume (µl)	10	4.508 ± 0.416	1,824,047 ± 174,420	1.201 ± 0.108	8,745 ± 866
	20	4.486 ± 0.041	3,780,085 ± 66,215	1.109 ± 0.018	8,973 ± 264
	30	4.425 ± 0.256	5,647,185 ± 338,672	0.914 ± 0.091	6,702 ± 534

injection volumes, the retention time, peak area, and number of theoretical plates showed significant variations (> 5% in repeat injections), which could affect the reproducibility of the quantification process. The poor repeatability at both low and high volumes suggests instability in signal response—possibly due to insufficient signal at low volume and detector saturation at high volume [21]. In addition, as the injection volume increased, the number of theoretical plates decreased. Therefore, to ensure peak stability, prevent tailing, and maintain reproducibility, an injection volume of 20 µl was selected.

The optimal parameters for the analysis were as follows: Shimadzu LC-2030C 3D HPLC system equipped with a PDA detector set at 230 nm, a Gemini 5 µm C18 110 Å LC column (250 × 4.6 mm), column temperature of 30°C, flow rate of 1.2 ml/minute, and injection volume of 20 µl, with a mobile phase consisting of acetonitrile and water (60:40, v/v).

3.2. Sample processing procedures

3.2.1. Sonication method

The results of the investigation on the sonication method are summarized in Table 4. As the sonication time increased, the detected amount of DTX in the sample also increased, reaching a peak at 100 minutes with 30.02%. However, extended sonication led to excessive heating, which compromised further experimentation. In addition, none of the samples met the required content criteria, indicating that the sonication method is unsuitable for extracting DTX from B@D in nano-injectable formulations. In some cases, prolonged ultrasonication led to a reduction in peak area compared to shorter sonication times. This may be attributed to partial degradation of DTX induced by extended high-energy cavitation, which can generate localized heating and reactive species, compromising drug stability. These findings suggest that optimal sonication conditions should maximize extraction efficiency while minimizing potential drug degradation. The sonication method, although commonly used for drug extraction, proved ineffective in this case [22].

Table 4. Assay results of sample according to ultrasonic time.

No.	Ultrasonic time (minutes)	Retention time (minutes)	Peak area	Total weight of DTX found (%)	Recovery rate (%)
1	20	4.505	692,282	0.1603	16.03
2	40	4.501	256,653	0.2281	22.81
3	60	4.497	116,985	0.2590	25.90
4	80	4.494	87,976	0.2823	28.23
5	100	4.497	68,029	0.3002	30.02

3.2.2. Redox method (biodegradation of BPMO)

For the redox method involving the biodegradation of BPMO, the solvent selectivity was assessed by extracting DTX using different solvents, as shown in Figure 1. The chromatogram of the test sample extracted with DMSO revealed a large peak in the 2–3 minute retention time range, indicating that DMSO extracted impurities that interfered with the quantification. DMSO extracted only 36.7% of DTX, the lowest recovery among the solvents tested. Ethanol, though providing a chromatogram similar to the standard sample, had a recovery rate of 52.43%, which was insufficient for accurate quantification. In contrast,

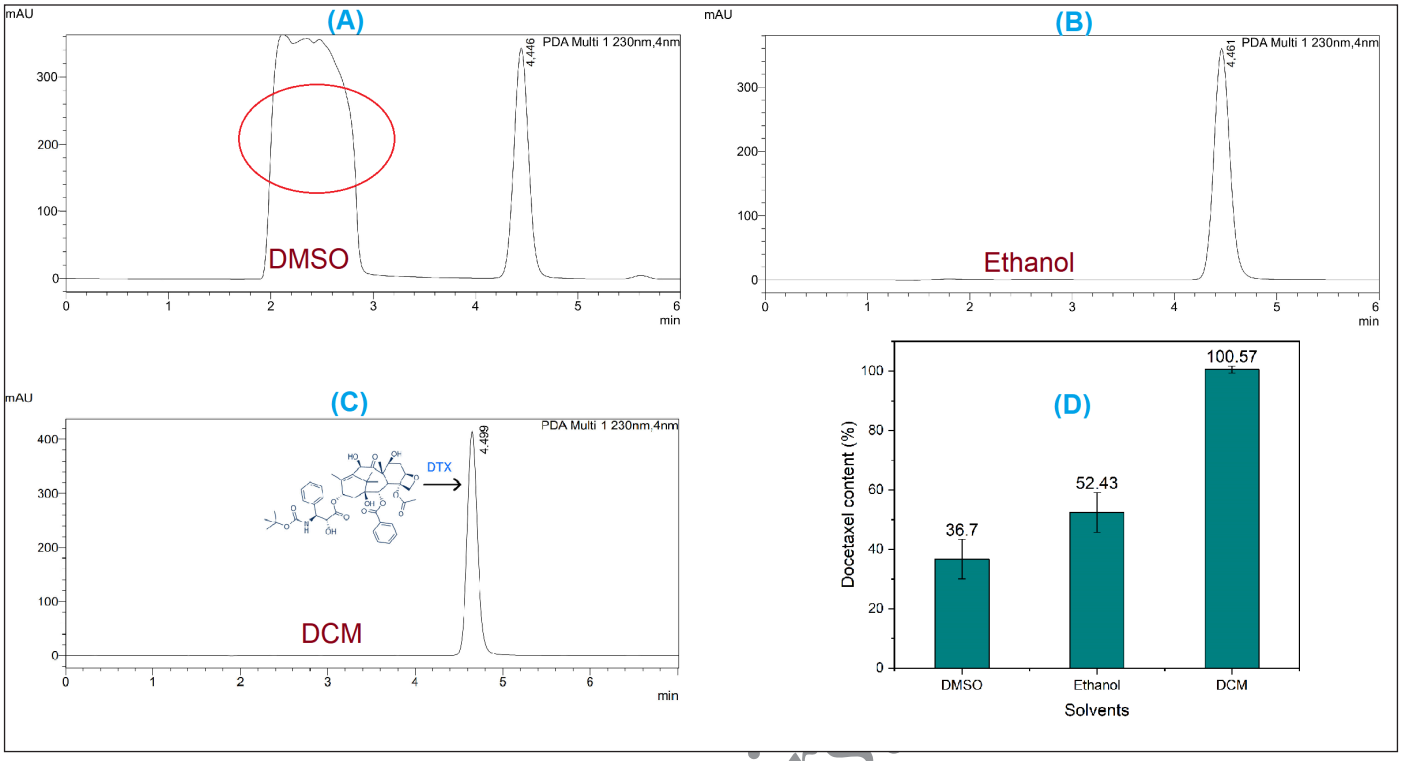

Figure 1. HPLC chromatograms of the test sample after extraction with (A) dimethyl sulfoxide (DMSO), showing additional non-DTX peaks; (B) absolute ethanol; and (C) dichloromethane (DCM), with clear identification of the docetaxel (DTX) peak. (D) Comparative docetaxel content (%) recovered from the three solvents, expressed as mean ± SD (n = 3). DCM exhibited the highest recovery efficiency (>100%), confirming its suitability as the optimal extraction solvent.

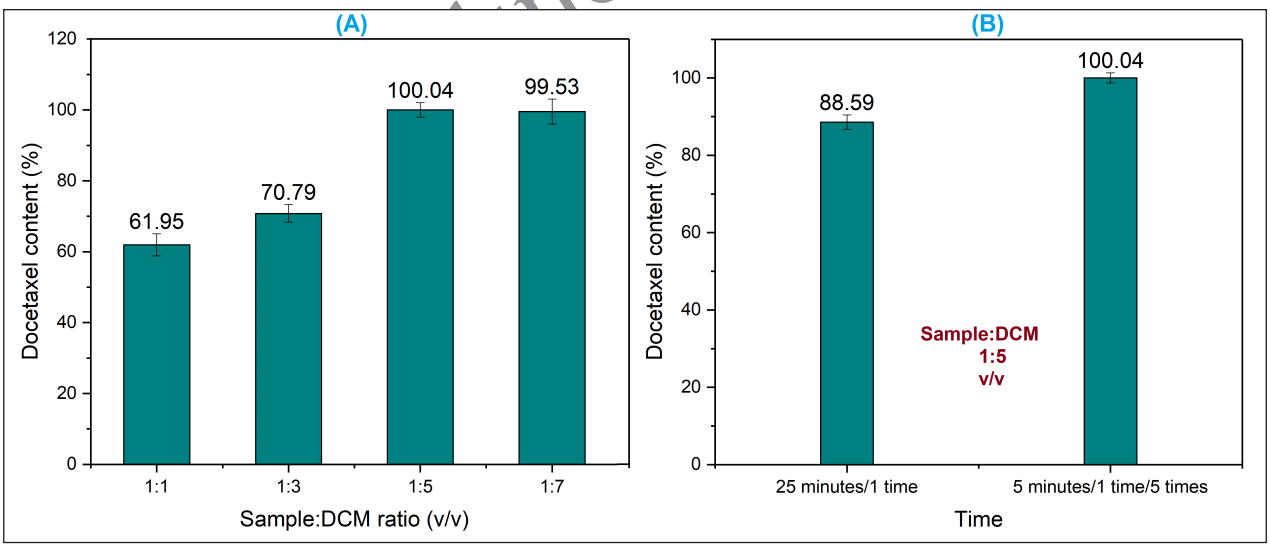

Figure 2. (A) Docetaxel content obtained from different sample/solvent ratios. (B) Docetaxel content obtained by varying extraction time.

DCM provided a clean baseline and recovered 100.57% of DTX, demonstrating complete extraction efficiency.

The effect of solvent ratio and extraction time on DTX recovery was further explored, as presented in Figure 2. Increasing the solvent-to-sample ratio resulted in higher DTX recovery, reaching a maximum at a 1:5 ratio, with no further increase at a 1:7 ratio. Therefore, a sample:DCM ratio of 1:5 was chosen for further investigation. Regarding extraction time, a single 25-minute extraction resulted in only 88.59% DTX recovery, whereas 5-minute extractions achieved 100.04% recovery. Thus, the optimized extraction method involved 5-minute extractions using a 1:5 sample:DCM ratio. The 5 × 5-minute extraction protocol produced significantly higher recovery than the 1 × 25-minute protocol (Welch's $t = -8.73$, df≈3.55, $p = 0.0016$). 95% CI: 1 × 25-minute [84.94; 92.25] versus 5 × 5-minute [97.99; 102.10].

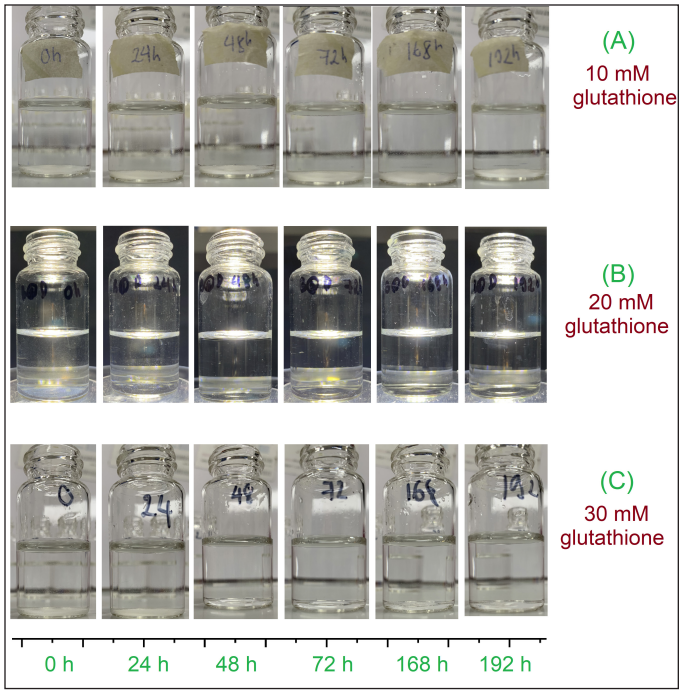

Figure 3. Visual appearance of B@D injection samples after degradation with GSH at (A) 10 mM concentration; (B) 20 mM concentration; and (C) 30 mM concentration in PBS 7.4.

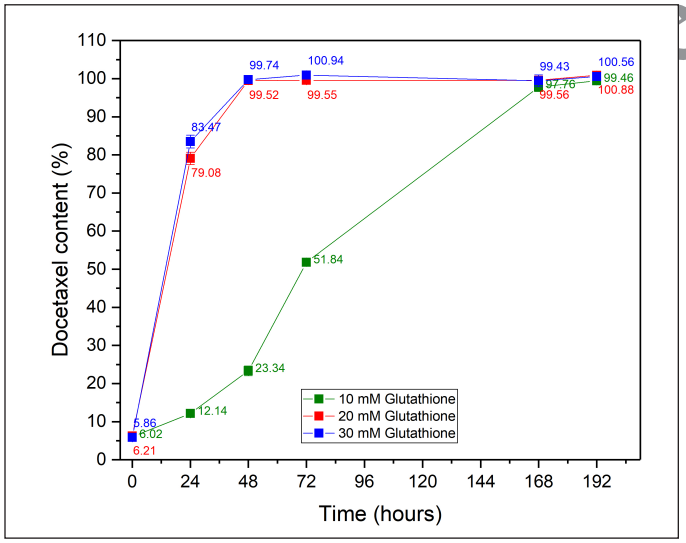

Figure 4. Quantification results of injection samples according to degradation time and GSH concentration.

Recovery differed significantly across ratios ($F(3,8)=141.94$, $p = 2.82 \times 10^{-7}$). Ratios 1:5 and 1:7 both outperformed 1:1 and 1:3 (all p_adj≤0.0036), with no significant difference between 1:5 and 1:7 (p_adj = 1.0000).

In the degradation study, B@D injectable samples were treated with GSH in PBS pH 7.4 at different concentrations. Visual observations, as shown in Figure 3, revealed that at a 10 mM GSH concentration, B@D degradation was minimal for the first 72 hours. The samples began to degrade only after 168 hours, showing weak degradation at this concentration (Fig. 4). At 20 mM and 30 mM GSH concentrations, the samples showed clear signs of degradation starting from 48 hours, with DTX content greater than 99.0% (99.52% at 20 mM and 99.74% at 30 mM) after 48 hours. The results indicated that a GSH concentration of 20 mM was suitable for the quantification process, allowing the sample to reach complete degradation within 48 hours.

Accordingly, the redox-based degradation approach using GSH in PBS pH 7.4 offered a much more viable solution [16]. The tetrasulfide linkages within BPMO are susceptible to cleavage under reductive conditions, enabling effective breakdown of the carrier and subsequent release of DTX. GSH concentrations of 20 mM and 30 mM both facilitated near-complete degradation within 48 hours, providing a practical timeframe for analytical workflows. This biocompatible and selective degradation mechanism supports both efficiency and reproducibility. At 48 hours, GSH concentration had a strong effect on recovery ($F(2,6)=4585.14$, $p = 2.80 \times 10^{-10}$). Both 20 mM and 30 mM GSH produced significantly higher recovery than 10 mM (p_adj≤8.13×10^{-7}), with no difference between 20 mM and 30 mM (p_adj=1.0000).

Solvent selection further optimized the extraction process. Among the tested solvents, DCM exhibited superior performance with complete recovery of DTX and no chromatographic interference, unlike DMSO and ethanol, which yielded low recovery rates and poor chromatographic profiles. The use of DCM also aligns with previous findings related to the extraction of structurally similar compounds such as paclitaxel [6,17]. Moreover, increasing the DCM-to-sample ratio improved extraction efficiency, likely due to enhanced solubilization of DTX. A 1:5 (v/v) sample-to-solvent ratio was determined to be optimal for consistent and efficient extraction.

One-way ANOVA showed a significant effect of solvent on DTX recovery ($F(2,6)=110.69$, $p = 1.84 \times 10^{-5}$). DCM yielded significantly higher recovery than DMSO (p_adj=0.0086) and ethanol (p_adj=0.0157), while the difference between ethanol and DMSO was not significant after multiplicity adjustment (p_adj=0.1335). 95% CI: DCM [97.70; 103.44], ethanol [35.86; 68.99], and DMSO [20.18; 53.22].

Overall, the optimized sample preparation method—incorporating biological degradation with GSH followed by DCM extraction—provides a reliable and reproducible protocol for quantifying DTX in complex nano-injectable systems.

In conclusion, the optimized procedure for degrading the nano-injectable B@D samples for DTX quantification involves dispersing 01 ml of the drug in 50 ml of PBS (pH 7.4) containing 20 mM GSH, stirring continuously at 400 rpm for 48 hours at 37°C. Sonication for 30 minutes is performed every 24 hours. Following degradation, DCM is added to the sample at a 1:5 ratio (v/v), and the mixture is shaken at 700 rpm for 10 minutes, then centrifuged at 6,000 rpm at 20°C to collect the DCM phase. This extraction process is repeated five times, with the extracts combined, evaporated to dryness, and reconstituted in a 5 ml 50:50 ACN:water solution for analysis by HPLC. Although the current protocol (48 hours of GSH-mediated degradation followed

by five sequential solvent extractions) provided the highest recovery and repeatability for B@D, it may be considered laborious for routine pharmaceutical QC applications. Future investigations could focus on streamlining the process by exploring faster degradation triggers, such as enzymatic digestion or alternative redox agents with higher reactivity but minimal impact on DTX stability, or by modulating temperature or pH to accelerate BPMO matrix breakdown. Preliminary observations suggest that reducing the extraction cycles from five to three still maintains recovery above 97%, which could significantly improve time efficiency without compromising analytical accuracy. It should be noted that two

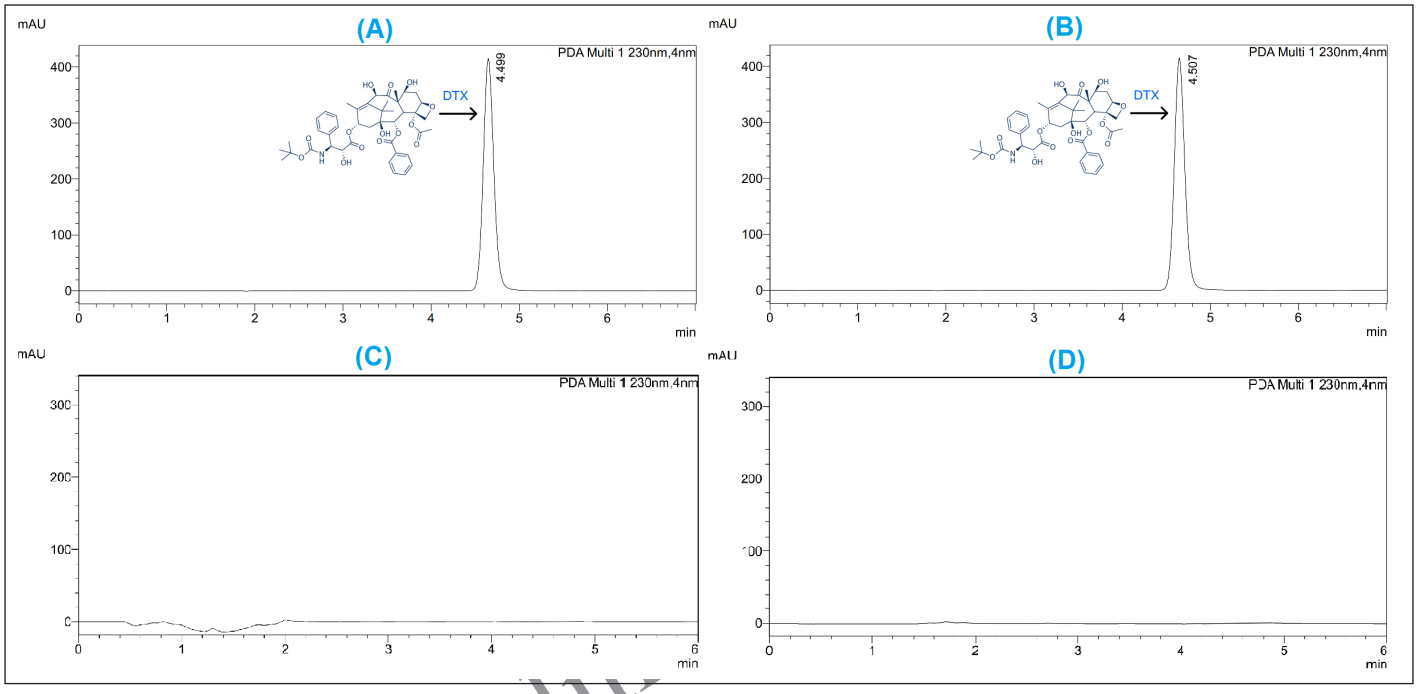

Figure 5. Chromatograms of samples: (A) Docetaxel standard; (B) Test sample; (C) Placebo; and (D) Blank sample.

Table 5. Results of system suitability and linearity assessment. Linearity was validated in the range of 60%–140% (0.12–0.28 mg/ml), while an additional concentration point at 0.32 mg/ml (160%) was also included to demonstrate extended linearity.

No.	Experiments	Retention time (minutes)	Peak area	Asymmetry factor (A_S)	Number of theoretical plates
1	System suitability	4.507	3,859,047	1.109	8,925
2		4.475	3,818,457	1.090	9,000
3		4.514	3,756,611	1.125	8,965
4		4.558	3,807,213	1.100	8,985
5		4.470	3,756,914	1.118	8,940
6		4.470	3,756,798	1.095	8,990
Average		4.499	3,792,507	1.106	8,968
RSD (%)		0.71	1.03	1.12	1.05

		DTX (mg/ml)	Retention time (minutes)	Peak area	Asymmetry factor (A_S)	Number of theoretical plates
1	Linearity	0.12	4.468 ± 0.577	2,278,633 ±1,927	1.095 ± 0.022	8,950 ± 279
2		0.16	4.486 ± 0.065	3,112,457 ± 58,732	1.115 ± 0.021	9,020 ± 281
3		0.2	4.520 ± 0.040	3,820,314 ± 79,456	1.102 ± 0.010	8,970 ± 293
4		0.24	4.497 ± 0.025	4,653,758 ± 93,214	1.110 ± 0.015	9,000 ± 328
5		0.28	4.480 ± 0.060	5,408,130 ± 97,876	1.108 ± 0.018	8,960 ± 369
6		0.32	4.487 ± 0.078	6,127,485 ± 99,789	1.121 ± 0.025	8,876 ± 245

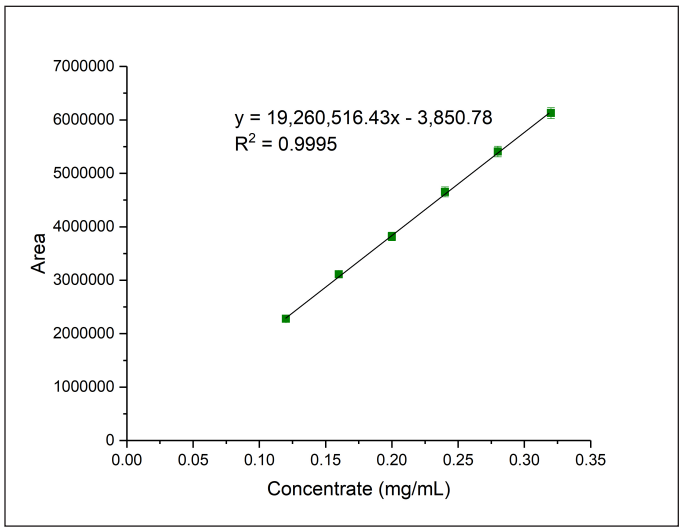

Figure 6. Graph representing the correlation between DTX concentration and peak area.

different extraction approaches were evaluated during method development: an ultrasonication-assisted extraction and a redox-triggered B@D degradation. Among these, the GSH-induced degradation method with supportive ultrasonication (centrifugation at 6,000 rpm) was selected as the optimized procedure and is the one described in the Methods section.

Although direct morphological evidence (e.g., SEM, TEM, and FTIR) was not obtained in the current study, several indirect observations strongly indicate complete BPMO degradation under the applied conditions (20 mM GSH, 48 h). These include (i) complete loss of sample opacity with only fine particulate residues, (ii) consistent >99% recovery of DTX after extraction, (iii) absence of interfering peaks from degradation products in the HPLC chromatograms, and (iv) consistency with previously reported TEM evidence of BPMO disintegration under similar GSH treatment [6,16]. Future work will include direct morphological and particle size characterization to further substantiate these findings.

3.3. Validation results of the analytical procedure

3.3.1. Specificity

Figure 5 shows the chromatograms of the standard sample, test sample, placebo, and blank used in the specificity validation. The chromatogram of the standard sample exhibits a well-defined and sharp DTX peak. No interfering peaks were observed in the placebo and blank samples at the retention time of the DTX peak. The chromatogram of the test sample displays the Docetaxel peak at the same retention time as the standard sample, confirming that the presence of excipients and BPMO (after degradation) does not interfere with the quantification of the active ingredient. Peak purity was further evaluated using the PDA detector at 230 nm, in accordance with ICH Q2(R^2) guidelines, and the Docetaxel peak was found to be spectrally homogeneous without co-eluting peaks. The method, therefore, demonstrates specificity.

Table 6. Comparative performance of selected analytical methods for DTX quantification in nano-matrices.

Parameter	This study (BPMO@ DTX; HPLC–UV)	Azren Aida *et al.* [23] (Palm-based nanoemulsion; HPLC–UV)	Rafiei *et al.* [24] (PLGA/PLGA-PEG NPs; LC–MS/MS) [24]
Linearity Range (mg/ml)	0.12–0.32	0.0,625–1.0	0.125–16.0 (PLGA); 0.125–8.0 (PLGA-PEG)
R^2	0.9,995	0.9,999	> 0.996
LOD (mg/ml)	0.0,049	0.00,988	0.0000,625
LOQ (mg/ml)	0.0,148	0.02,993	0.000,125
Accuracy (%)	98–102	93.3–99.9	LLOQ accuracy < ~13% dev.
Precision (%RSD)	≤ 2	0.8–1.9	CV ≤ ~11.6
Recovery (%)	~100 (extraction), 99.60 (spike)	93.3–99.9	— (no data founded)

Table 7. Accuracy assessment results.

No.	Percentage of DTX standard (%)	Weight of DTX (mg)	Peak area	Amount of DTX found (mg)	Recovery rate (%)
		0.811	3,052,968	0.805	99.26
1	80%	0.792	3,041,590	0.802	101.26
		0.824	3,026,420	0.798	96.84
		1.02	3,830,432	1.010	99.02
2	100%	0.994	3,784,922	0.998	100.40
		1.01	3,860,772	1.018	100.79
		1.19	4,551,008	1.20	100.84
3	120%	1.23	4,626,859	1.22	99.19
		1.21	4,532,046	1.195	98.76
Average					99.60
RSD(%)					0.78

3.3.2. System suitability and linearity

The summary results of the system suitability and linearity validation were presented in Table 5. Table 5 summarizes the results of the system suitability and linearity validation. The RSD for retention time and peak area was below 1.5%, and the asymmetry factor ranged from 0.8 to 1.2, indicating the system is suitable for the chosen quantification method. The regression equation for DTX concentration and peak area, shown in Figure 6, is y = 19,260,516.429x—3,850.781 with R^2 = 0.9995, indicating strong linearity. The correlation between peak area and concentration is statistically significant (F = 7656.06, $p < 0.05$), confirming a strong linear relationship within the concentration range of 0.12 mg/ml to 0.32 mg/ml.

The limit of detection (LOD) and limit of quantification (LOQ) were calculated according to ICH Q2(R^2) as LOD = 3.3

Table 8. Results of precision and repeatability.

No.	Experiments	DTX concentration in sample (mg/ml)	Peak area	DTX concentration found (mg/ml)	Percentage (%)
1		1.02	3,805,874	1.0,035	98.38
2			3,857,293	1.0,171	99.71
q3			3,792,518	1.0,000	98.04
4			3,861,942	1.0,183	99.83
5	Repeatability		3,788,165	0.9,989	97.93
6			3,839,730	1.0,125	99.26
Average (n = 6)					98.86
RSD (%) (n = 6)					0.83
1		1.01	3,781,025	0.9,970	98.71
2			3,812,142	1.0,052	99.52
3			3,854,896	1.0,165	100.64
4			3,808,963	1.0,043	99.44
5	Intermediate precision		3,898,245	1.0,279	101.77
6			3,843,177	1.0,134	100.33
Average (n = 12)					99.46
RSD (%) (n = 12)					1.12

× (σ/S) and LOQ = 10 × (σ/S), where σ is the standard deviation of the intercept and S is the slope of the calibration curve. The calculated values were LOD = 0.0049 mg/ml and LOQ = 0.0148 mg/ml, indicating that the method is suitable for quantifying DTX even at low concentrations. The validated working range (0.12–0.32 mg/ml) was selected to encompass the expected DTX concentration in B@D injectable formulations after extraction; samples outside this range can be diluted or concentrated to fall within the validated range.

Comparative context with published assays: to contextualize our validation outcomes, we summarized representative methods that quantified DTX in nano-matrices (Table 6). Relative to a palm-based nanoemulsion HPLC–UV assay, our method achieves a comparable or lower LOQ (0.0148 *vs.* 0.0299 mg/ml) while maintaining excellent linearity (R^2 = 0.9995) and ICH-compliant accuracy/precision. As expected, LC–MS/MS assays validated directly in PLGA/PLGA-PEG nanoparticle matrices report substantially lower LOQs (down to 0.000125 mg/ml), but require specialized instrumentation and are less accessible for routine QC. These comparisons support the suitability of our simple ACN:water HPLC–UV method for day-to-day analysis of B@D injectables [23, 24]. Collectively, these data indicate that the proposed method balances performance and practicality for B@D.

3.3.3. Accuracy

Table 7 presents the results on accuracy, indicating that the recovery rates of the samples fall within the acceptable range of 98%–102%. Therefore, the quantification method for DTX in nano-injectable B@D samples using HPLC meets the accuracy criteria.

3.3.4. Precision—repeatability—determination range

Table 8 provides the results for precision and repeatability. The sample content remained within the acceptable range of 98%–102%, with both the %RSD (n =6) and the combined %RSD (n = 12) being less than 2%. Thus, the method satisfies the precision and repeatability requirements for quantitative analysis of DTX in nano-injectable DTX samples. The validated detection range for the quantification of DTX in the B@D nano-injectable formulation was determined based on the linearity study and method sensitivity. The method showed acceptable linearity, accuracy, and precision across the concentration range of 0.12–0.32 mg/ml, corresponding to 60%–160% of the target assay concentration. This range fully encompasses the expected sample concentrations during routine analysis and meets the ICH Q2(R^2) acceptance criteria for assay/content determination. It should be noted that the 1 mg/ml concentration reported for accuracy and precision corresponds to the nominal 100% assay level of the test solution. After preparation and dilution, this concentration lies within the validated linearity range (0.12–0.32 mg/ml), ensuring consistency across all validation parameters.

The validation results confirmed that the developed HPLC method meets essential analytical criteria, including accuracy, precision, specificity, linearity, and a well-defined detection range [18]. These parameters collectively demonstrate that the method is reliable and reproducible for the quantification of DTX. Compared to the current USP method for DTX injection [14], which typically requires more complex solvent systems and chromatographic conditions, this method offers a simplified yet effective alternative. By employing a binary ACN:water mobile phase and standard chromatographic settings, the method is not

only cost-effective but also accessible for routine use in basic laboratory environments. This practicality, combined with its validated performance, makes the method highly applicable for quality control of nano-injectable DTX formulations.

Robustness and Ruggedness: The robustness of the method was evaluated by introducing minor deliberate variations in chromatographic parameters, including flow rate (±0.1 ml/minute), column temperature (±2°C), and detection wavelength (±2 nm). These changes did not significantly affect retention time, peak area, or theoretical plates ($p > 0.05$), indicating that the method is robust under small operational variations. Ruggedness testing was performed by two different analysts on separate days using different HPLC instruments. The %RSD values for retention time and peak area were consistently <2%, demonstrating good inter-analyst and inter-instrument reproducibility.

System suitability: System suitability parameters were within the acceptable limits for all test runs. The tailing factor for the DTX peak was <1.5, the number of theoretical plates exceeded 5,000, and the resolution between the DTX peak and the nearest excipient-related peak was >2.0. These results confirm that the method meets the requirements for reliable quantification of DTX in B@D formulations.

4. CONCLUSION

This study successfully established a reliable and validated HPLC method for quantifying DTX in the nano-injectable B@D formulation. By applying a redox-triggered degradation approach using 20 mM GSH in PBS (pH 7.4), the method effectively disrupted the stable BPMO–DTX complex, enabling complete drug recovery within 48 hours. The developed chromatographic method fulfilled the ICH Q2(R^2) validation requirements, including specificity (with peak purity evaluation), linearity across 0.12–0.32 mg/ml (60%–160% of the nominal concentration), accuracy, precision (repeatability and intermediate precision), robustness, and range. These results confirm that the method is reliable and well-suited for application in future research and formulation development involving B@D nano-injectables. Moreover, this validated method can be applied for routine quality control of BPMO-based DTX nanoformulations in pharmaceutical development.

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6. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

7. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

8. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

9. DATA AVAILABILITY

The authors declare that all the data supporting the findings of this study are available within the paper.

10. PUBLISHER'S NOTE

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11. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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